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The Role Of Skin Resident Cd4 T Cells In Cutaneous Leishmaniasis

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Abstract
Cutaneous leishmaniasis is a disease characterized by highly inflammatory, sometimes disfiguring lesions that nonetheless spontaneously resolve, resulting in robust protection against reinfection. It has been known for decades that a type 1 CD4+ T cell response is critical for both primary parasite control and subsequent protection against reinfection. However, all attempts to artificially reproduce this response have failed, suggesting there may be a gap in our understanding of the memory CD4+ T cells generated by natural infection. In this work, we use a healing mouse model of cutaneous leishmaniasis to explore the role of skin-associated CD4+ T cells in this disease. We demonstrate for the first time that tissue-resident memory (TRM) CD4+ T cells are generated by leishmania infection, are long-lived, and are distributed throughout the skin. We next investigate whether or not these cells contribute to immune protection, and find that leishmania-specific TRM cells can guide the rapid recruitment of circulating T effector cells to the site of challenge, enhancing control of infection. We go on to identify another, novel mechanism of TRM cell-driven protection, in which leishmania-specific TRM cells orchestrate the rapid infiltration and activation of inflammatory monocytes, leading to robust early control of disease even in the absence of circulating T cells. Finally, we explore the requirements for the establishment of TRM cell populations in cutaneous leishmaniasis, with the ultimate goal of understanding how to harness these cells in a potential vaccine.

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ABSTRACT

THE ROLE OF SKIN RESIDENT CD4 T CELLS IN CUTANEOUS LEISHMANIASIS

Nelson D. Glennie

Phillip Scott, Ph.D.

Cutaneous leishmaniasis is a disease characterized by highly inflammatory, sometimes disfiguring lesions that nonetheless spontaneously resolve, resulting in robust protection against reinfection. It has been known for decades that a type 1 CD4+ T cell response is critical for both primary parasite control and subsequent protection against reinfection. However, all attempts to artificially reproduce this response have failed, suggesting there may be a gap in our understanding of the memory CD4+ T cells generated by natural infection. In this work, we use a healing mouse model of cutaneous leishmaniasis to explore the role of skin-associated CD4+ T cells in this disease. We demonstrate for the first time that tissue-resident memory (T_{RM}) CD4+ T cells are generated by leishmania infection, are long-lived, and are distributed throughout the skin. We next investigate whether or not these cells contribute to immune protection, and find that leishmania-specific T_{RM} cells can guide the rapid recruitment of circulating T effector cells to the site of challenge, enhancing control of infection. We go on to identify another, novel mechanism of T_{RM} cell-driven protection, in which leishmania-specific T_{RM} cells orchestrate the rapid infiltration and activation of inflammatory monocytes, leading to robust early control of disease even in the absence of circulating T cells. Finally, we explore the requirements for the establishment of T_{RM} cell populations in cutaneous leishmaniasis, with the ultimate goal of understanding how to harness these cells in a potential vaccine.

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CHAPTER 1: Introduction

1.1 Leishmaniasis

Leishmaniasis, a disease caused by many different species of the intracellular protozoan parasite leishmania, remains a serious global public health problem. According to estimates, roughly 1 million new infections occur each year, and hundreds of millions more are at risk of infection (World Health Organization, 2017). The disease is transmitted by the sand fly, with endemic areas of disease falling predominantly in equatorial regions that support the arthropod vector. As the global climate continues to change, it is predicted that the range of the sand fly will expand further, putting even more people at risk for infection (Iba et al., 2013).

The life cycle of leishmania is relatively simple (Kaye and Scott, 2011). The parasites are transmitted by sand flies that harbor the flagellated promastigote form of the parasite. When feeding, sand flies deposit promastigotes in the skin, and phagocytic cells rapidly take up the parasites. While the initial cells that become infected include neutrophils, macrophages, and monocytes, it is primarily within macrophages that the parasites survive and replicate as non-flagellated amastigotes. Surprisingly, unlike other intracellular parasites, such as toxoplasma or *Trypanosoma cruzi*, leishmania survives and replicates within the phagolysome, having evolved the ability to persist in an environment meant to destroy most microbes. After several rounds of replication, the amastigotes rupture the infected cell and invade other neighboring macrophages. The parasites differentiate back into promastigotes when taken up by sand flies, continuing the infectious cycle.

Leishmaniasis is characterized by diverse clinical entities ranging from self-healing cutaneous lesions, to severe, disfiguring chronic lesions, and even causes fatal disease in its visceral form. Drug treatment for leishmaniasis is notoriously poor and, despite decades of research, there is no vaccine for this disease, creating a continuing need for the development of new therapies. Antibodies play no protective role, and indeed may exacerbate disease (Miles et al., 2005). CD8+ T cells may play a protective role in certain contexts (Uzonna et al., 2004; Müller et al., 1994), but have also been implicated in the exacerbation of the disease (Crosby et al., 2014; Novais et al., 2013). In contrast, CD4+ T cells are the cells required for control, specifically IFNγ produced by these cells, which activates infected macrophages to kill the parasites. Thus, the inability to develop a successful vaccine is largely due to a lack of knowledge on how to generate long-lived memory CD4+ T cells (Kaye and Scott, 2011). Studying the CD4+ T cell subsets involved in protective immunity against leishmaniasis is an opportunity with the potential to improve prophylactic treatments for this disease.

1.2 Protective mechanisms in cutaneous leishmaniasis

The production of reactive oxygen species (ROS) during phagocytosis, called the respiratory burst, is an innate response that allows macrophages and neutrophils to kill microbes before the development of an adaptive response. Those microbes that survive innate killing become pathogens that require a greater response for elimination, which can be induced by the action of IFNγ. This IFNγ response leads to increased production of ROS, as well as activation of inducible nitric oxide synthase, leading to the release of nitric oxide (NO). Leishmania parasites are killed by both ROS and NO, but different myeloid cells may be better able to kill by one mechanism than the other. For example,

inflammatory monocytes exhibit a high level of ROS and can kill leishmania without being activated, while macrophages require IFNγ activation for killing (Novais et al., 2014; Goncalves et al., 2011). Under some circumstances neutrophils cooperate with macrophages to kill the parasites (Novais et al., 2009), although in other situations neutrophils appear to inhibit parasite control (Afonso et al., 2008). Further complicating the matter, although it is clear that NO is required for the ability of mouse macrophages to kill leishmania, the role of NO in human cells remains controversial (Novais et al., 2014; Carneiro et al., 2016; Vouldoukis et al., 1995). Nonetheless, in both mice and humans IFNγ is absolutely required to activate infected phagocytes to eliminate parasites, leading to control of the disease.

Many cell types can produce IFNγ, including NK cells, ILCs, γδ T cells, CD4+ T cells and CD8+ T cells. However, while all IFNγ producing cells may contribute to an effective response, only CD4+ T cells can transfer resistance against leishmania infection to reconstituted RAG-/- mice (Belkaid et al., 2002a; b; Novais et al., 2013). The critical attribute of these cells appears to be their MHC restriction, since it was recently found that adoptive transfer of class II restricted, but double negative (CD4-, CD8-) T cells, also provides resistance to leishmania (Mou et al., 2014). Nevertheless, while not protective by themselves, other IFNγ producing cells can play important roles in augmenting protection. NK cells (and possibly other innate lymphoid cells) accelerate the development of a Th1 response when CD4+ T cells are being primed in the draining lymph nodes (Laouar et al., 2005; Prajeeth et al., 2011; Scharton and Scott, 1993). Similarly, following a low dose challenge, IFNγ from CD8+ T cells promotes the CD4+ Th1 response (Uzonna et al., 2004). Nonetheless, CD4+ T cells remain the predominant source of protective IFNγ, and will be the focus of this work.

1.3 Complexities of the immune response to leishmaniasis

There are some unusual aspects of the immune response to leishmania that are worth noting. First, it is important to consider that control of the parasites and the development of disease are often dissociated. This is most evident in *L. braziliensis* patients with mucosal disease, where the severe lesions are chronic and debilitating, but contain few parasites. Conversely, while RAG-/- mice are unable to control parasite replication, they develop only minor swelling at the site of infection. Adoptive transfer of CD4+ T cells to these immunocompromised mice is sufficient to allow for parasite control, but may lead to the development of larger lesions, although these lesions generally resolve, depending on the leishmania species (Belkaid et al., 2002b; Novais et al., 2013; Soong et al., 1997). On the other hand, when RAG-/- mice are reconstituted with CD8+ T cells they not only fail to control the parasites, but also develop a severe inflammatory response, as well as the development of metastatic lesions (Belkaid et al., 2002b; Novais et al., 2013). This is a surprising result, since under certain circumstances CD8+ T cells can promote protection (Uzonna et al., 2004; Muller et al., 1993; Müller et al., 1994). It turns out that whether CD8+ T cells promote resistance or increased disease relates directly to whether they primarily produce IFNγ or are cytolytic, as increased cytolytic activity promotes a pathologic inflammatory response (Novais et al., 2013; Santos et al., 2013). Since we will focus on IFNγ-producing CD4+ T cells that are rarely associated with immunopathology, this work will emphasize mechanisms required for parasite control.

Although there is a shared pathway for resistance to leishmania that requires IFNγ, there are many different strains and species of the parasites, adding additional complexity to the disease. These different species can induce distinct immune responses, and have

differential sensitivities to macrophage killing. This is particularly true of species in South America, which can produce chronic infections in mice normally resistant to *L. major* (McMahon-Pratt and Alexander, 2004). One example is *L. amazonensis*, which induces a chronic disease in C57BL/6 mice (Khouri et al., 2009). *L. amazonensis* parasites induce a weaker CD4+ Th1 response than *L. major* infection, and are also able to resist killing by activated macrophages that can kill *L. major* (Scott et al., 1983). Even within the same leishmania species, different strains can lead to diverse outcomes following infection (Anderson et al., 2005). From this, one might conclude that vaccines and the memory T cells they generate might be effective against one species or strain of leishmania, but less effective against another. On the other hand, several studies have shown cross-protection between species, providing some evidence that a single vaccine may work for different leishmania parasites, and even protect against the visceral form of the disease (Romano et al., 2015; Mou et al., 2015). In all our studies we will use the Friedlin strain of *Leishmania major* as a representative cutaneous leishmania infection, but anticipate that our findings may have broader relevance despite inevitable species-specific differences.

1.4 Protective CD4+ T cells in cutaneous leishmaniasis

Following resolution of a primary infection, mice and humans are highly resistant to reinfection with leishmania. As aforementioned, this resistance is primarily dependent upon CD4+ T cells, although other IFNγ-producing cells can contribute to immunity in some situations (Muller et al., 1993; Müller et al., 1994; Okwor et al., 2014). For the interest of this work, we will focus on what is known about the CD4+ T cell subsets

involved in providing protection, specifically effector T cells (T_{Eff}), effector memory T cells (T_{EM}), and central memory T cells (T_{CM}).

The strong resistance observed in healed mice is dependent in part on the persistence of a low number of parasites (Uzonna et al., 2001; Belkaid et al., 2002a). Thus, the few parasites that are left after the resolution of a primary infection maintain a pool of true CD4+ T_{Eff} cells that can rapidly respond to a challenge. T_{Eff} cells from immune mice, as defined by low expression of CD62L, have been shown to transfer protection against disease due to their ability to home to the site of infection and rapidly produce cytokine (Zaph et al., 2004). Recently, this population of short-lived T_{Eff} cells has been even more thoroughly characterized (Peters et al., 2014). These cells phenotypically resemble the CD4+ T_{Eff} cells generated after viral infection (Marshall et al., 2011) in that they express high levels of Ly6C and T-bet, produce IFNγ at a high frequency upon activation, and, as previously demonstrated, can transfer rapid protection (Peters et al., 2014). Unfortunately, these cells fail to persist in the absence of antigen (Zaph et al., 2004; Peters et al., 2014), and are thus difficult to target for vaccine-induced protection despite being highly protective in natural infection.

In contrast to T_{Eff} cells, memory T cells are defined by their ability to be maintained in the absence of antigen once an infection is cleared. Memory CD4+ T cells have classically been divided into two subsets, central memory (T_{CM}) and effector memory (T_{EM}) cells, based on surface marker expression, tissue tropism, proliferative capacity, and effector function (Sallusto et al., 1999). Central memory T cells express CD62L and CCR7, which allow them to efficiently traffic through the blood and lymph nodes. Upon restimulation, T_{CM} cells rapidly proliferate and thus provide a pool of differentiated,

antigen-specific cells to combat a secondary infection. In contrast, T_{EM} cells lack CD62L and CCR7, circulate through blood and non-lymphoid tissues, and exhibit effector functions, such as cytokine production and cytotoxicity, upon restimulation. T_{EM} cells are often distinguished from closely related T effector cells by their ability to persist after antigen is cleared, but can also be identified by IL-7R expression on CD8+ T cells (Kaech et al., 2003), and additionally by the absence of Ly6C expression on CD4+ T cells (Marshall et al., 2011).

In the context of leishmania infection, since the parasites persist chronically, we must rely exclusively on phenotypic and functional markers to distinguish memory cell subsets. To this end, it was once a question of whether or not memory CD4+ T cells would exist in leishmaniasis at all. However, there is indeed a population of CD62L high CD4+ T cells that demonstrate T_{CM} cell activity by rapidly proliferating to generate T_{Eff} cells upon restimulation with leishmania antigen (Zaph et al., 2004). Further, when transferred to naïve recipients these CD62L high cells can provide protection, albeit delayed in comparison to CD62 low T_{Eff} cells (Zaph et al., 2004). Importantly, these CD62L high cells can persist and transfer protection in the absence of antigen, as demonstrated using a replication-deficient parasite (Zaph et al., 2004). Intriguingly, antigen-specific $CD62L^{+}CCR7^{+}$ cells can be identified within the first couple weeks of infection, indicating that T_{CM} and T_{Eff} cells are generated concurrently. This cell fate decision appears to be affected by the timing of antigen experience and the number of proliferative cycles a cell has gone through (Colpitts and Scott, 2010). T_{CM} cells are not only a critical component of secondary protection, but also an appealing target for vaccination since they have the ability to proliferate to provide a robust pool of T_{Eff} cells, and because they are maintained in the absence of persistent antigen.

Not surprisingly, the role for T_{EM} cells in leishmania infection is less defined, as it becomes difficult to distinguish these cells from T_{Eff} cells maintained by the chronic infection. Nonetheless, there is evidence that leishmania infection generates a population of antigen experienced $CD62L^{-}IL\text{-}7R^{+}Ly6C^{-}$ cells (Colpitts et al., 2009), that fit the T_{EM} cell phenotype. However, the role for these cells in protection remains unclear as they may not be able to migrate to the infection site or proliferate effectively following challenge (Peters et al., 2014). For simplicity, we will group circulating T cell responses into T_{Eff} or T_{CM} cells in leishmaniasis, as they are the most easily distinguishable and most relevant cell types.

1.5 Resident Memory T cells

In addition to circulating T_{CM} and T_{Eff} cells, a new subset of non-migratory memory T cells that resides in non-lymphoid tissue has been classified as resident memory T cells (T_{RM}). These cells were first discovered in the gut (Kim et al., 1999; Masopust et al., 2001) and lung (Hogan et al., 2001a; b) around the same time T_{CM} and T_{EM} cells were first being defined (Sallusto et al., 1999), but were initially thought by many to be T_{EM} cells in transit through the tissue because of their phenotypic similarity to T_{EM} cells (Masopust et al., 2001). Although some distinctions from T_{EM} cells suggested that these tissue cells might be a unique cell subset (Kim et al., 1999; Hogan et al., 2001a; Masopust et al., 2006), it was not until more recently that transplantation (Gebhardt et al., 2009) and parabiotic surgery (Jiang et al., 2012) confirmed that T_{RM} cells in tissue could persist in the tissue at disequilibrium from cells in circulation, and in the absence of any antigen exposure (Casey et al., 2012).

T_{RM} cells have now been identified, characterized, and demonstrated to promote protection in such diverse tissues as the gut (Kim et al., 1999; Masopust et al., 2001, 2006; Bergsbaken and Bevan, 2015; Bergsbaken et al., 2017), lung (Hogan et al., 2001a; b; Cauley et al., 2002; Ely et al., 2003; Teijaro et al., 2011; Laidlaw et al., 2014; Wu et al., 2013; Purwar et al., 2011), skin (Clark et al., 2006; Gebhardt et al., 2009; Jiang et al., 2012; Gaide et al., 2015; Watanabe et al., 2015; Collins et al., 2016), brain (Wakim et al., 2010, 2012; Steinbach et al., 2016), female reproductive tract (Schenkel et al., 2013; Schenkel, 2014; Shin and Iwasaki, 2012; Iijima and Iwasaki, 2014), liver (Tse et al., 2013), salivary glands (Hofmann and Pircher, 2011; Thom et al., 2015), and even secondary lymphoid organs (Schenkel et al., 2014b). While initial studies described T_{RM} cells that provided protection against acute viral infections, (Gebhardt et al., 2009; Teijaro et al., 2011; Jiang et al., 2012; Schenkel et al., 2013), these findings have now been extended to a number of bacterial (Sakai et al., 2014; Bergsbaken and Bevan, 2015; Bergsbaken et al., 2017), and even parasitic (Tse et al., 2013) infections.

Many of the initial T_{RM} studies were performed on CD8+ T cells (Kim et al., 1999; Hogan et al., 2001a; Masopust et al., 2001, 2006; Gebhardt et al., 2009; Jiang et al., 2012), but it is now clear that CD4+ cells also form T_{RM} cell populations (Teijaro et al., 2011; Iijima and Iwasaki, 2014; Laidlaw et al., 2014; Collins et al., 2016). While these CD4+ and CD8+ T_{RM} cells share some important characteristics in that they reside in tissue and enhance early protection, distinctions have been identified regarding their location, migratory properties, and function. In skin, it has been found that HSV-specific CD8+ T_{RM} cells are associated with the epidermis, while CD4+ cells are more likely to be found in the dermis (Gebhardt et al., 2011). Moreover, while both CD4+ and CD8+ T cells are seen to associate with hair follicles, CD8+ T cells show an increased dependence on IL-

15 signaling (Adachi et al., 2015), while CD4+ cells have been showed to demonstrate clustering behavior both in the skin (Collins et al., 2016) and the female reproductive tract (Iijima and Iwasaki, 2014) where they rely on myeloid cell networks for maintenance. CD4+ cells are also much more likely to be migratory within tissue (Debes et al., 2005; Gebhardt et al., 2011; Bromley et al., 2012; Collins et al., 2016), while a majority of CD8+ T cells present in tissue appear to be more universally sessile and resident (Mackay et al., 2013). This suggests that there may be more heterogeneity in CD4+ cells in tissues, and thus more caution may be necessary when studying putative CD4+ T_{RM} cells to ensure that they are indeed tissue-resident.

CD4+ and CD8+ T_{RM} cells appear to have both unique and overlapping protective functions when they become activated in tissue. Perhaps the most obvious function for CD8+ T_{RM} cells is to mediate protection through direct cytotoxic killing of infected cells. Indeed, activation of CD8+ T_{RM} cells has been associated with expression of cytolytic molecules perforin and granzyme in a variety of contexts (Kim et al., 1999; Masopust et al., 2006), and in at least one case protection against brain infection has shown to be perforin dependent (Steinbach et al., 2016). While CD4+ T_{RM} cells are unlikely to mediate protection through direct cytotoxicity, cytokine production has also been shown to be critical for protection in a number of different cases (Jiang et al., 2012; Shin and Iwasaki, 2012; Schenkel et al., 2013; Laidlaw et al., 2014; Steinbach et al., 2016), both for CD4+ and CD8+ T_{RM} cells. This cytokine production can lead to many different downstream effects, including the maintenance of other protective tissue cell subsets (Iijima and Iwasaki, 2014; Laidlaw et al., 2014), the recruitment of circulating effector T and B cells (Schenkel et al., 2013), or the maturation of local innate cell types such as NK cells and DCs (Schenkel, 2014; Ariotti et al., 2014). In all cases, T_{RM} cells provide

enhanced protection by acting in a rapid, antigen specific manner at the site of infection. However, T_{RM} cells are not always beneficial, and various T_{RM} cell subsets have also been implicated in cutaneous T cell lymphoma (Watanabe et al., 2014), psoriasis (Cheuk et al., 2014), and asthma (Hondowicz et al., 2015).

In order to better study T_{RM} cells, and to better define the factors involved in their generation and maintenance, it would be useful to identify a unique, universal signature that defines tissue residency. Unfortunately, while there appear to be some core similarities between different T_{RM} cell subsets, there also appears to be a significant degree of heterogeneity. CD103 and CD69 are the most commonly used markers of T_{RM} cells (Masopust et al., 2001; Schön et al., 1999; Mackay et al., 2015; Teijaro et al., 2011), but both have proven inconsistent markers (Anderson et al., 2014; Watanabe et al., 2015). Down regulation of receptors associated with tissue egress such as CCR7, S1P1R, and the associated transcription factor KLF-2 are more consistently associated with T_{RM} cells (Bromley et al., 2012; Skon et al., 2013), but these changes are not necessarily unique to T_{RM} cells, and can be difficult to measure. More recent global analysis has revealed that CD8+ T_{RM} from a variety of tissues do have an overlapping gene signature and display a wide array of migration, adhesion, and activation markers (Mackay et al., 2013), and may even have a common transcriptional program (Mackay et al., 2016). However, the scope of these studies remains limited; for example they do not examine CD4+ T_{RM} cells at all. There are bound to be tissue and pathogen-specific differences in different T_{RM} cell populations, and defining these factors will be important for studying these T_{RM} cells.

1.6 Delayed type hypersensitivity and skin inflammation

A delayed type hypersensitivity response (DTH) is a hallmark of protective, cell mediated immunity against a number of different diseases such as tuberculosis, leprosy, and leishmaniasis (Bretscher, 1992; Howard et al., 1980). Classically, this response is thought to be associated with antigen-recognition by pathogen specific T cells, and the response is delayed because it takes days for cells to respond and infiltrate the tissue, leading to the associated swelling and redness. Intriguingly, two recent studies have called into question the role of circulating T cells in the contact hypersensitivity model, a related model of skin inflammation (Adachi et al., 2015; Gaide et al., 2015). In both cases, T_{RM} cells proved critical for driving the contact hypersensitivity response, while having circulating T cells alone in the absence of T_{RM} cells led to a delayed or otherwise dampened response (Adachi et al., 2015; Gaide et al., 2015). Whether or not a similar phenomenon occurs in the delayed type hypersensitivity response associated with protection against leishmaniasis remains to be determined.

The lymphocyte infiltration associated with a DTH response is regulated by a cascade of surface molecule adhesions between T cells expressing molecules such as VLA-4, P and E selectin ligands, and LFA-1 and endothelial cells expressing their binding partners in VCAM-1, P and E selectin, ICAM-1, and PECAM-1, all of which are upregulated during inflammation (Muller, 2013; Iijima and Iwasaki, 2015). While the rules governing T cell infiltration into inflamed tissue are reasonably well defined, it remains unclear what processes are involved in T cell entry into uninflamed tissue, although it is evident that in some infection models this entry does occur, resulting in the establishment of T_{RM} cells that are widely distributed and not confined to a local site (Masopust et al., 2001; Jiang et al., 2012). Developing models of T_{RM} cell formation in which there is significant

infiltration of T cells into uninflamed sites will allow for better study of this issue, which is important because generating T_{RM} cells throughout tissue will likely be critical for inducing broad protection.

1.7 Summary

The CD4+ T cell response generated during a primary leishmania infection is highly protective against reinfection, but this response has yet to be successfully reproduced in the absence of natural infection, suggesting some deficiency in our understanding of the cells involved in protection. During a primary infection (Fig. 1), antigen-specific effector T cells are primed in the draining lymph node, proliferate, migrate to the site of infection, and mediate protection. Concurrently, a population of long-lived CD62L+ T_{CM} cells is generated that can further feed into the T_{Eff} cell response. T_{Eff} and T_{CM} cells are maintained once infection is resolved and are important for secondary protection, but whether or not skin-associated T_{RM} cells might form during leishmania infection and further augment protection is unknown. Here, we investigate the role of skin-resident CD4+ T cells during leishmania infection. T_{RM} cells can provide protection in a number of different ways, so we also explore mechanisms by which leishmania T_{RM} cells might mediate protection (Fig. 2). The goal of these studies is to gain a better understanding of the requirements for the generation and maintenance of the T cells involved in a protective immune response to leishmania in the skin, with the hope that this knowledge can be leveraged to develop better vaccines against the disease.

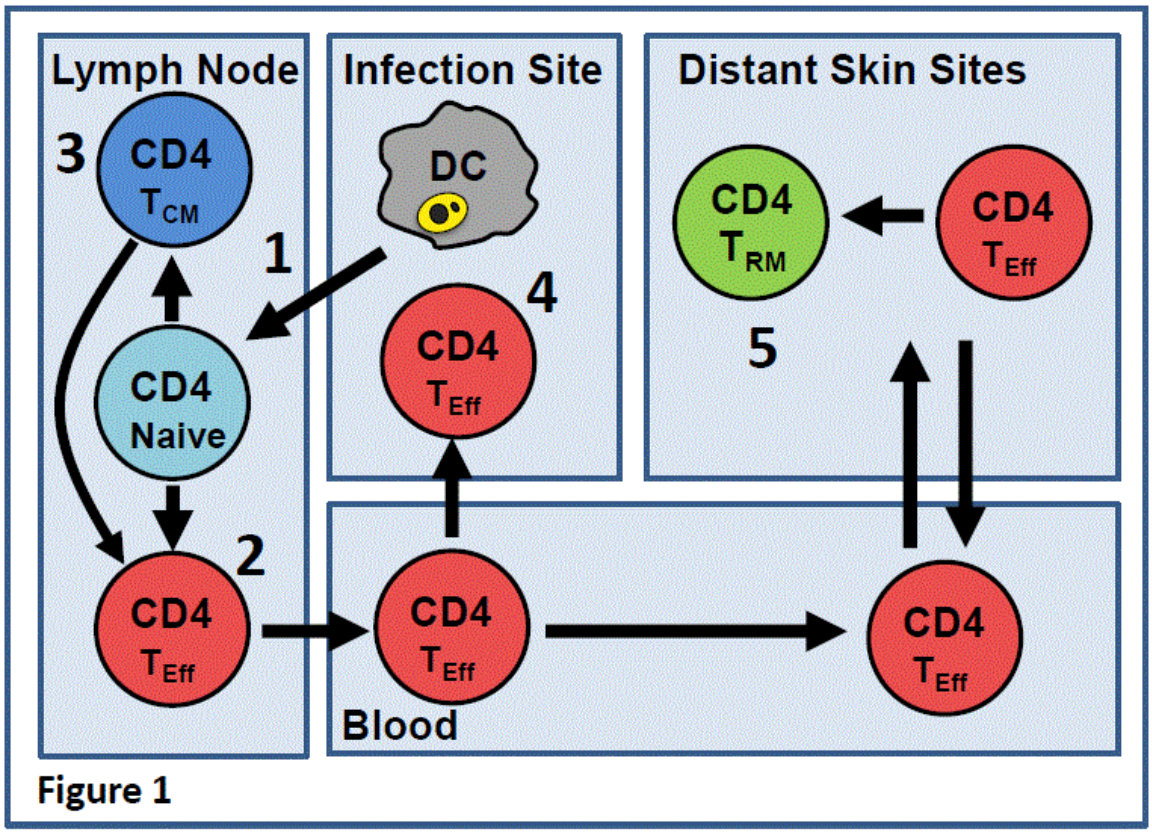


Figure 1. T cell responses following infection with leishmania. Leishmania parasites are deposited in the skin by infected sand flies and rapidly invade phagocytic cells, including dendritic cells (DC). DCs migrate from the skin to the draining lymph node (1) where they stimulate naïve T cells, which proliferate and differentiate into effector T (T_{Eff}) cells (2) or central memory (T_{CM}) cells (3). T_{Eff} cells leave the lymph nodes and migrate to the site of infection (4), where they produce IFNγ leading to parasite control. Some of the T_{Eff} cells may be retained at the site of infection, or migrate to distant skin sites, where they could become resident memory T (T_{RM}) cells (5).

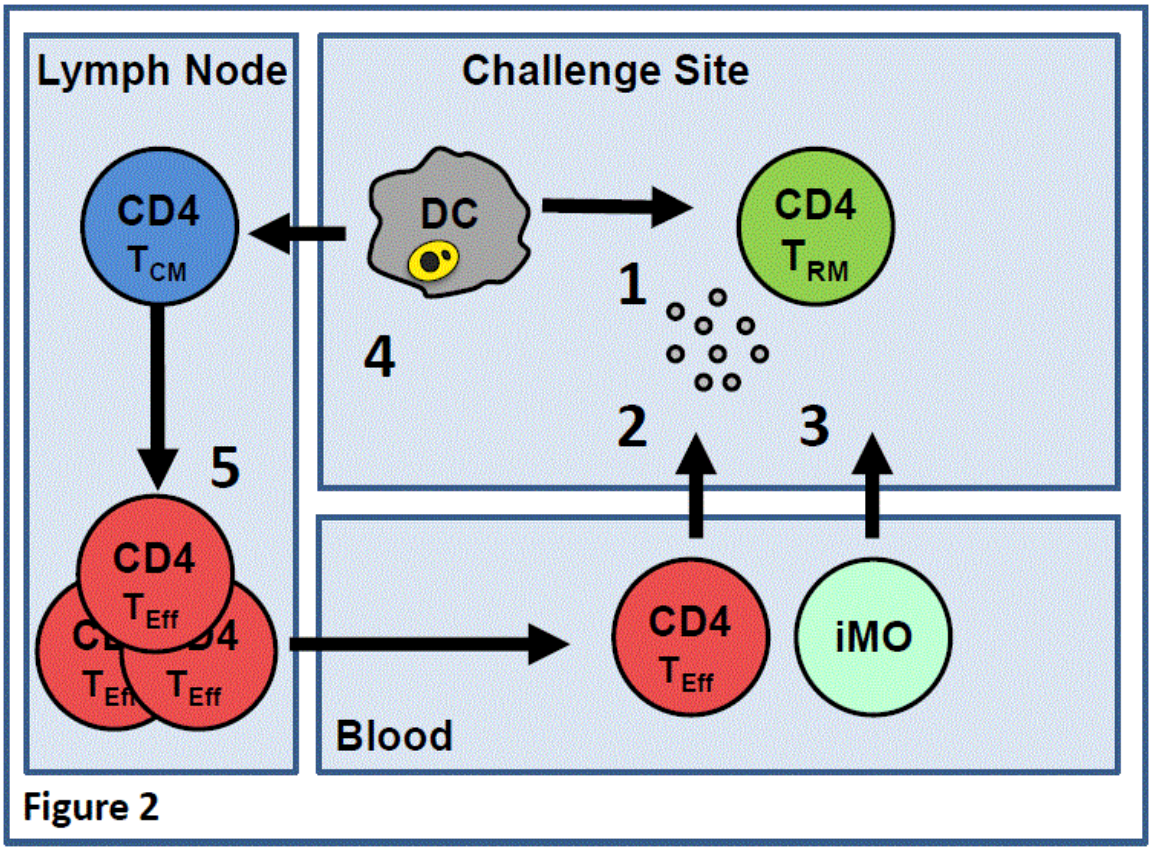


Figure 2. Potential T_{RM} cell responses following a secondary infection with leishmania. During a secondary infection, T_{RM} cells are poised to respond rapidly. Upon activation, T_{RM} cells could function in a number of ways including (1) the production of cytokines, (2) the recruitment of T_{Eff} cells or (3) phagocytic cells such as inflammatory monocytes (iMO), or (4) activating DCs to migrate from the skin to the draining lymph node to stimulate T_{CM} cells to become (5) T_{Eff} cells.

CHAPTER 2: Skin Resident Memory $CD4^+$ T Cells Enhance Protection Against *Leishmania major* Infection

2.1 Abstract

Leishmaniasis causes a significant disease burden worldwide. Although Leishmania-infected patients become refractory to reinfection following disease resolution, human vaccines have not yet achieved effective immune protection. While circulating

Leishmania-specific T cells are known to play a critical role in immunity, the role of memory T cells present in peripheral tissues has not been explored. Here we identify a population of skin-resident leishmania-specific memory CD4+ T cells. These cells produce IFNγ, and remain resident in the skin when transplanted by skin graft onto naïve mice. They function to recruit circulating T cells to the skin, resulting in better control of the parasites. Our findings are the first to demonstrate that CD4+ T_{RM} cells are generated in response to a parasitic infection, and indicate that protective immunity to leishmania, and thus the success of a vaccine, may depend on generating both circulating and skin-resident memory T cells.

2.2 Introduction

The development of effective vaccines for several intracellular microbial pathogens, such as mycobacteria, toxoplasma, plasmodium, and leishmania, remains an elusive goal. Despite substantial efforts to define the mechanisms required for resistance, to develop new adjuvants, and to identify protective antigens, the long-lived cellular immunity generated in response to infection has not been recapitulated by vaccination. To address this problem in leishmaniasis, we have focused on defining the memory T cells that mediate infection-induced immunity.

C57BL/6 mice show robust immunity to reinfection following resolution of a primary *Leishmania major* infection (referred to here as 'immune mice'), providing a useful model to interrogate the factors that might contribute to a successful vaccine. Previous studies have shown that immune mice contain circulating CD4+ T cells with effector, effector memory, and central memory phenotypes (Scott et al., 2004; Colpitts et al., 2009;

Colpitts and Scott, 2010). Each of these T cell subsets likely plays a role in resistance to reinfection, with the effector subsets rapidly migrating into tissues to provide protection and central memory T cells proliferating in the draining lymph node to provide a pool of new effector cells. However, while adoptive transfer of either effector or central memory T cells to naïve mice enhances immunity to reinfection (Zaph et al., 2004), neither subset alone or in combination provides the same level of protection as that seen in intact immune animals.

In addition to circulating memory T cells, an additional memory T cell subset resides in the tissues as resident memory T cells (T_{RM}) (Kim et al., 1999; Masopust et al., 2001; Hogan et al., 2001a; Clark et al., 2006; Gebhardt et al., 2009; Wakim et al., 2010; Hofmann and Pircher, 2011; Jiang et al., 2012; Mackay et al., 2012; Schenkel et al., 2013). Several studies have described T_{RM} cells that mediate immunity to acute viral infections, such as vaccinia, herpes simplex, influenza, and lymphocytic choriomeningitis virus (Gebhardt et al., 2009; Teijaro et al., 2011; Jiang et al., 2012; Schenkel et al., 2013). These T_{RM} cells can be found in the gut, brain, lung, and skin (Kim et al., 1999; Clark et al., 2006; Wakim et al., 2010; Teijaro et al., 2011), and their location allows them to respond immediately to control a challenge infection without the delay associated with the mobilization of circulating T cells. Additionally, T_{RM} cells can promote rapid recruitment of effector cells from the circulation (Schenkel et al., 2013) and induce antigen-independent innate immunity (Schenkel et al., 2014a; Ariotti et al., 2014), therby accelerating and amplifying resistance to infections.

CD8+ T_{RM} cells are fairly well characterized, but less is known about CD4+ T_{RM} cells. Nevertheless, recent studies using Kaede-Tg mice to facilitate tracking of T cells in the

skin indicate that a population of CD4+ T cells appear to be skin-resident under homeostatic conditions (Bromley et al., 2012). In addition, CD4+ T_{RM} cells in the lung and vaginal mucosa have been reported to enhance resistance to influenza and herpes simplex virus respectively (Hogan et al., 2001b; Teijaro et al., 2011; Iijima and Iwasaki, 2014), and a population of human tissue-resident CD4+ T cells remain in the skin after circulating T cells have been depleted (Clark et al., 2012). However, the potential role of CD4+ T_{RM} cells in establishing resistance to chronic parasitic infections such as *L. major* is virtually unknown.

Here we identify for the first time a tissue resident population of CD4+ T cells that seed the skin following *L. major* infection. These cells are observed in skin sites distant from the primary infection site soon after infection, and persist long term in immune mice. They produce IFNγ in response to stimulation with *L. major,* and during a secondary challenge act as sentinels to rapidly recruit circulating memory cells, resulting in enhanced protection against reinfection. Thus, our results suggest that this previously unidentified population of memory CD4+ T cells is instrumental in protection against leishmania parasites and should now be considered during vaccine development.

2.3 Materials and Methods

Mice

C57BL/6 mice were purchased from the National Cancer Institute (Fredericksburg, MD). *Ifng/Thy1.1* knock-in mice were provided by Dr. Casey Weaver (University of Alabama at Birmingham). All mice were maintained in a specific pathogen-free environment at the University of Pennsylvania Animal Care Facility. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory

Animals of the National Institutes of Health, and the protocol was approved by the Institutional Animal Care and Use Committee.

Parasites

L. major (Friedlin) parasites were grown in Schneider's insect medium (GIBCO) supplemented with 20% heat-inactivated FBS, 2mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Metacyclic enriched promastigotes were used for infection (Späth and Beverley, 2001). Mice were infected with 2×10^6 *L. major* in the left ear and sacrificed 8-64 weeks after infection.

Antibodies

For flow cytometry analysis anti-CD45 AF700, anti-CD45.2 APC-eF780, anti-CD45.1 eF480, anti-CD90.2 APC, anti-CD69 FITC, anti-CD103 PE, anti-CD90.1 PE-Cy7, anti-FoxP3 PE, anti-CD11b FITC (eBioscience), anti-CD4 PE TexasRed (Invitrogen), anti-CD8b PerCp/Cy5.5, and anti-IFNγ PE-Cy7 (Biolegend) were used. For in vivo CD4+ depletion mice received intraperitoneal (i.p.) injections of 250 µg GK1.5 on days -5 and -2 before challenge. For intravascular staining mice were injected with 3 µg anti-CD45.2 AF780 (eBioscience) 3 minutes before they were euthanized by CO_2.

Skin Preparation

For ear preparation, dorsal and ventral layers of the ear were separated and incubated in RPMI (Gibco) with 250 µg/mL Liberase TL (Roche) for 90 minutes at 37°C in 5% CO_2. Cells within auricular skin were dissociated using a 40 µm cell strainer (BD Pharmingen) and resuspended in RPMI media containing 10% FBS. For flank preparation, flank skin was shaved using an electric trimmer equipped with a two-hole precision blade (Wahl)

and treated with depilating agent (Nair) for 1 minute. A section of dermis was excised, then minced with a sterile scalpel blade into ~2mm sections. Flank sections were incubated in RPMI containing 250 µg/mL Liberase TL for 120 minutes with vortexing every 30 minutes. The resulting solution was passed through a 40 µm cell strainer and resuspended in complete RPMI (cRPMI) supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 55 µM 2-Mercaptoethanol.

Bone marrow derived dendritic cell (BMDC) restimulation

BMDCs were generated by culturing C57BL/6 bone marrow in GM-CSF supplemented cRPMI for 7-11 days. BMDCs were then harvested and infected for 5-8 hours with stationary phase *L. major* at a ratio of 10:1 in the presence of 1 µg/ml CpG and LPS. Infected BMDCs were incubated at a ratio of 1:5 with 10^6 skin homogenate cells in 24 well plates overnight 12-16 hours, in the presence of 5 µg/ml BFA (eBioscience) for the last 4 hours when intracellular IFNγ staining was performed. Samples were harvested, stained with monoclonal antibodies, and data collected on an LSR cytometer.

Parasite titration

Parasite burden from ear and flank skin was calculated by serial dilution (1:10 and 1:5 respectively) in 96-well plates incubated at 26°C. The number of viable parasites was calculated from the highest dilution at which parasites were observed 7 days into culture.

Skin grafts

Donor skin was prepared under sterile conditions from naïve and immune mouse flank skin by shaving, depilating, cleaning with chlorhexidine (Vetoquinol), then excising the skin using sterile instruments. 8mm biopsy punches (Miltex) were used to size donor

grafts, which were then placed onto a graft bed generated by a 6mm biopsy punch of mice anesthetized with isofluorene and treated with 0.1 mg/kg buprenorphine as a preemptive analgesic. Grafts were covered with non-adherent dressing (Adaptec) and held in place with Tegaderm (3M) wrapped circumferentially around the body of the mouse to provide protection against loss and trauma. Mice were monitored twice daily for the first 48 hours and daily for 7 days after grafting, given buprenorphine and rewrapped where necessary. In challenge experiments, grafted skin was injected intradermally with 2×10^6 metacyclic *L. major* 10-14 days after grafting.

Adoptive transfer

Single cell suspensions from spleen and lymph node were incubated 4 minutes at room temperature in the presence of 2.5 µM CFSE in the dark. The reaction was quenched with 10% FBS and resuspended at $5\text{-}10 \times 10^7$ cells/mL in PBS, then transferred intravenously (i.v.) in 400 µL volume into recipient mice.

LCMV infection

C57BL/6 mice were injected i.p. with 2×10^5 pfu LCMV Armstrong. LCMV immune donor mice were sacrificed at least 15 days after infection, single cell suspensions were generated from spleen and lymph node, CFSE labeled, and transferred i.v. into recipient mice.

Transcriptional profiling

Flank tissue sections were homogenized in RLT buffer using a rotor-stator. RNA was isolated using the RNeasy Plus kit (Qiagen). Biotin-labeled complementary RNA (cRNA) was generated using the TargetAmp-Nano amplification kit (Epicentre). RNA and cRNA

quality were assessed on a BioAnalyzer (Agilent). MouseRef-8 v2.0 Expression BeadChips (Illumina) were then hybridized with cRNA from 4 immune and 4 naïve mice challenged in the flank with *L. major*. Analysis was performed using the statistical computing environment R (v3.0.2), with RStudio and Bioconductor suite packages (v0.97; Boston, MA). Data have been deposited on the GEO database for public access (GSE# pending). GSEA (Subramanian et al., 2005) was performed with the Broad Institute's MSigDB (v4.0) to query the "C2: Canonical Pathways" collection, which contains 1320 gene sets.

Statistical analysis

Statistical analysis was performed with the Mann-Whitney test (two-sided t-test, paired or unpaired where applicable) in Prism software (GraphPad).

2.4 Results

L. major specific CD4+ T cells are present in skin distal from the primary infection site

C57BL/6 mice infected with *L. major* in the ear develop a lesion that resolves by 12 weeks. These mice are resistant to reinfection, and are referred to as immune mice. To determine if leishmania-specific T cells were present in skin sites distant from the primary infection site, we isolated cells from the uninfected ear (contralateral ear) and the flank. We also isolated cells from the primary infection site (primary ear) of immune mice, and from the ears and flank of naïve mice. The cells were incubated with or without *L. major* infected dendritic cells overnight and IFNγ production assessed as a measure of T cell activation. We utilized *Ifng/Thy1.1* knock-in mice in these experiments

(Harrington et al., 2008) so that we could use surface staining for Thy1.1 to sensitively detect transcription of the IFNγ gene. As expected, leishmania-responsive cells were absent in ear and flank skin from naïve mice, while approximately 25% of $CD4^+$ T cells from the primary ear of immune mice produced IFNγ in the presence of *L. major* infected DCs, likely due to a low level of persistent infection (Fig. 1*A*, Fig 1*B*). However, when we stimulated cells from two uninfected sites of immune mice, the contralateral ear and the flank skin, we also detected a population of CD4+ T cells that responded to *L. major* infected DCs (Fig. 1*A,* Fig 1*B*). These cells did not respond to uninfected DCs, demonstrating their specificity. In separate experiments, we used intracellular staining to confirm the production of IFNγ protein (Fig. 1*C*). A similar population of IFNγ responsive CD4+ T cells in immune mice was also recently observed (Peters et al., 2014). Importantly, after lesion resolution, no parasites could be detected in either the contralateral ear or the flank skin by culture, qRT-PCR for ribosomal RNA, or PCR for kinetoplast DNA (data not shown).

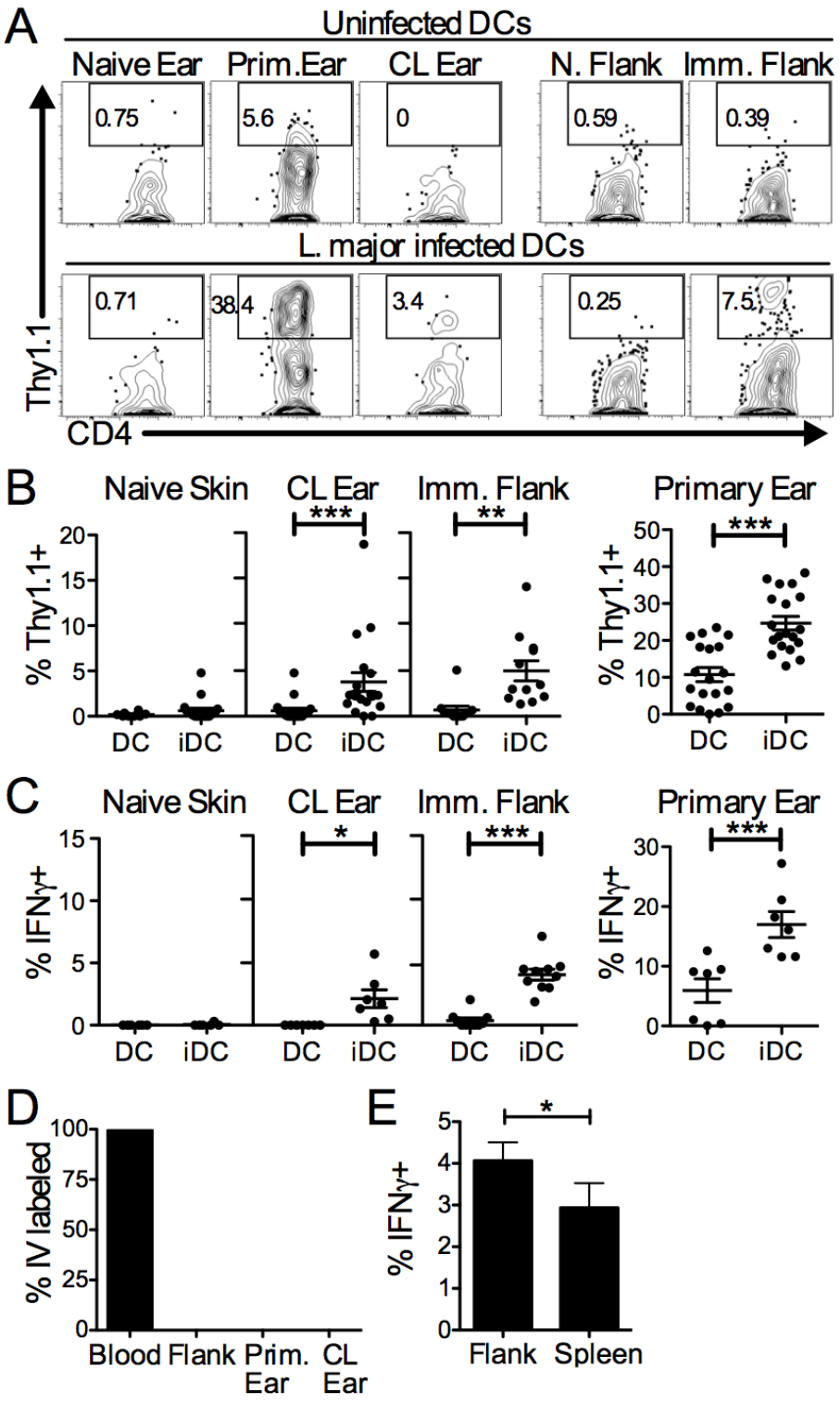


Figure 1. *L. major*-specific CD4+ T cells are present in skin distant from the primary infection site. Skin was collected from either naïve C57BL/6 mice (wild-type or IFNγ-reporter mice) or mice that had been previously infected with *L. major* in the left ear and resolved their lesions (referred to as immune mice).

Skin isolated from infected mice included the left ear (primary ear), contralateral ear (CL), and immune (Imm.) flank; skin from naïve mice (N.) included the ear and the flank. (A-C) Cells from the skin were incubated with either uninfected bone-marrow derived dendritic cells (DC) or BMDC that were infected with *L. major* (iDC), and 12 hours later IFNγ production in $CD4^+$ T cells (gated on live, CD45, CD90.2, CD4 cells) was assessed by expression of Thy1.1 (A, B) or by IFNγ intracellular staining (C). (A) Representative flow plots. (B, C) Each dot represents an individual mouse, and data are combined from at least 3 experiments. (D) FITC anti-CD45.2 antibody was injected iv into immune mice (15 wks post-infection) and 3 minutes later blood, flank, primary ear (Prim.) and contralateral (CL) ear were collected and incubated with infected BMDCs as above, and FITC staining assessed on CD4+ IFNγ+ cells. Data shown are from one representative experiment of 2 (n = 5 mice). (E) Cells isolated from the flank or spleen of immune mice were incubated with *L. major* infected BMDCs as above, and each bar represents the mean (± SEM) percent IFNγ producing CD4+ T cells detected by flow cytometry (gated on live, CD45, CD4 cells). Data shown are combined from 3 experiments (n = 10 mice per group). *, $P < 0.05$; **, $P < 0.01$; *** $P < 0.001$.

To determine if the leishmania-responsive cells we observed in the contralateral ear and flank of immune mice might be in the vasculature of the skin, we used a technique developed to distinguish resident and circulating T cells in tissues (Anderson et al., 2012). Immune mice were injected with FITC anti-CD45.2 antibody intravenously, and 3 minutes later the blood and skin were collected and assessed for responsiveness to *L. major*-infected dendritic cells. The IFNγ+ cells from the blood were FITC+, while cells from the skin were not, indicating that the leishmania-responsive cells were indeed contained within the skin, sequestered from circulation (Fig. 1*D*). Consistent with the potential for these cells to be resident in the skin, we found that >80% of the leishmania-responsive cells in the contralateral ear and flank were CD69+ and all of the cells were CCR7- (data not shown). Finally, when we compared the frequency of leishmania-responsive cells in the skin to the spleen, we found a significant enrichment in the skin, suggesting that there may be preferential retention of these cells in the tissue (Fig. 1*E*). Together, our results identify a previously unrecognized population of T cells distributed

broadly throughout the skin, positioned to act as a frontline defense against leishmania infection.

Leishmania-specific CD4+ T cells are resident in immune skin

To determine how long after infection *L. major*-specific CD4+ T cells could be detected in skin distant from the primary infection site, we incubated infected DCs with skin cells from immune mice at various time points after infection. *L. major*-specific CD4+ T cells persisted in skin sites distant from the primary infection site at every time point we observed, up to one year after resolution of disease (Fig. 2*A*). While these data confirmed that *L. major*-responsive CD4+ T cells are a persistent population in the skin, they did not distinguish whether these T cells were resident in skin or were instead being replenished from circulation.

To address this issue, we grafted uninfected flank skin from immune CD45.2 mice originally infected in the ear onto naïve CD45.1 recipients, and determined how long donor *L. major*-specific T cells remained in the grafts by quantifying the number of leishmania memory cells present within donor skin at the time of grafting (t = 0), and in harvested grafts 1, 2, and 4 weeks later. At all time points we were able to identify *L. major*-specific donor cells resident within the graft skin (Fig. 2*B*). The difference between the number of *L. major*-specific cells at the time of grafting and later time points was not significant but did trend towards a decrease, suggesting that some cells might recirculate as well. Regardless, leishmania-responsive CD4+ T cells were retained for at least 4 weeks in immune skin in the absence of circulating memory cells, and thus form a tissue-resident population.

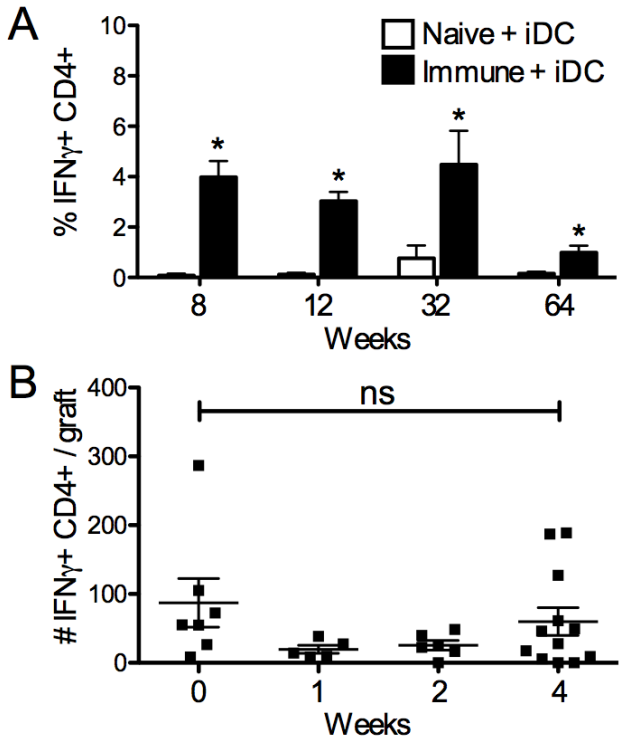


Figure 2. Leishmania-specific CD4+ T cells are resident in immune skin. (A) Flank skin was collected from naïve or immune mice at various time points after *L. major* infection, and IFNγ producing CD4+ T cells detected as in Fig. 1. Data shown represents the mean (± SEM) percent IFNγ producing CD4+ T cells detected by flow cytometry (n= 3-4 mice per time point). (B) Flank skin from immune mice (13-14 weeks post-infection) was grafted onto naïve mice, and at various times the grafts were harvested and IFNγ producing CD4+ T cells detected as in Fig. 1. Quantification of *L. major*-specific cells in skin grafts before grafting (time 0) and 1, 2, or 4 weeks later is shown. Data are combined from two separate experiments and each dot represents one mouse. *, $P < 0.05$.

L. major immune mice rapidly upregulate interferon and chemokine signaling following *L. major* challenge

To determine if *L. major*-specific T_{RM} cells could contribute to the immune response early after a challenge infection, we injected previously uninfected flank skin from naïve and immune mice with *L. major*, harvested the inoculated skin 12 hours later, and compared

gene transcript levels. We found 145 differentially regulated genes between naïve and immune skin (fold change ≥ 2, false discovery rate ≤ 0.05) (Fig. 3*A*). Strikingly, competitive gene set enrichment analysis revealed interferon signaling to be the most highly upregulated canonical signaling pathway in immune mice (Fig. 3*B*). Chemokine and chemokine receptor signaling were also among the most significantly enhanced gene sets (Fig. 3*C*).

To confirm that the transcriptional changes we observed were due to cells resident in immune skin and independent of any cells that might have been recruited, we isolated flank skin cells and incubated them with *L. major* in vitro. We assessed the transcriptional response of a select group of genes found to be upregulated by global transcriptional profiling using qRT-PCR and observed a significant increase in expression of several IFNγ responsive genes (Fig. 3*D*). Notably, CXCL9 and CXCL10 were highly expressed in restimulated flank skin both *in vitro* and *in vivo*. These two chemokines interact with CXCR3 on activated T cells to drive T cell recruitment (Nakanishi et al., 2009). These data, along with recent evidence in the literature, (Schenkel et al., 2013; Ariotti et al., 2014) led us to hypothesize that *L. major*-specific T_{RM} cells might function by activating a program to rapidly recruit circulating effector T cells to the site of infection after challenge.

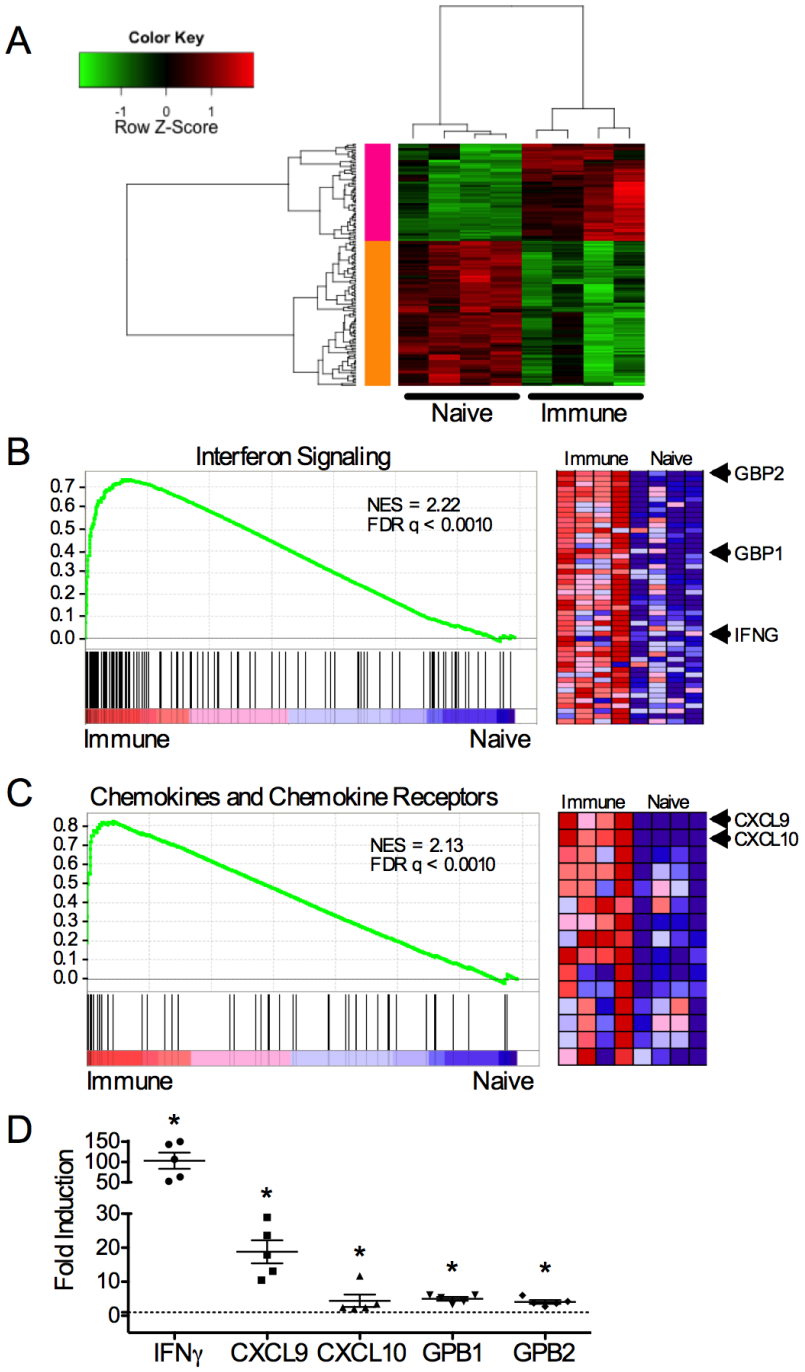
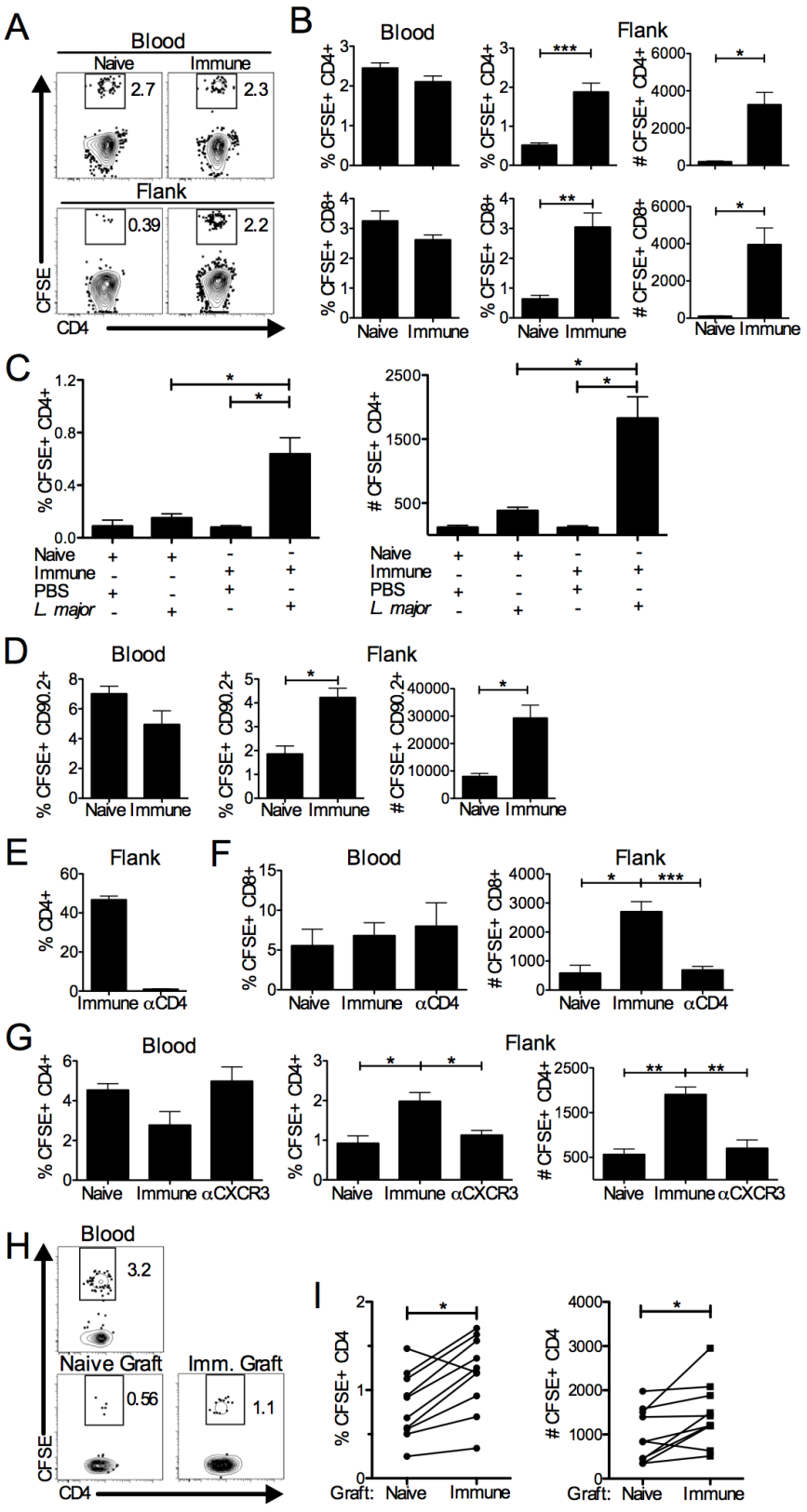


Figure 3. *L. major* immune mice rapidly upregulate interferon and chemokine signaling following challenge. Naïve or immune mice (20 wk post-infection) were infected in the flank with *L. major*, and the flank skin harvested 12 hours later for transcriptional profiling by microarray. (A) Heat map of 145 differentially expressed genes between 4 naïve and 4 immune mice challenged in the flank (fold change ≥ 2, false discovery rate ≤ .05). (B, C) Gene set enrichment analysis plot for gene sets "interferon signaling" (B) and "chemokines and chemokine receptors" (C). Heat maps show the top upregulated genes from the interferon-signaling gene set or chemokines and

chemokine receptors gene set between naïve and immune mice (red = high, blue = low). Normalized enrichment score (NES) with false discovery rate q-value as calculated by GSEA. (D) Cells were isolated from the flank skin of immune mice (20 weeks post-infection) were incubated with or without *L. major* parasites for 12 hours and gene expression assessed by qRT-PCR. Data shown are the fold induction of IFN-regulated genes following exposure to *L. major.* Each dot represents an individual animal, and is from one representative experiment of 2. *, $P < 0.05$.

Leishmania T_{RM} cells enhance T cell recruitment following *L. major* challenge

To compare the recruitment of effector T cells to an *L. major* challenge site with and without leishmania-specific T_{RM} cells, we transferred CFSE-labeled splenocytes from immune mice into naïve or immune recipients, infected the flank skin, and measured the number of cells recruited from the blood to the challenge site 20 hours later. Although equivalent pools of circulating cells were available in the blood of naïve and immune recipients, there was significantly enhanced recruitment of both $CD4^+$ and $CD8^+$ T cells to the skin of leishmania-immune animals (Fig. 4*A*, 4*B*). Increased recruitment was not a response to tissue injury alone, as PBS injection was insufficient to increase recruitment in immune animals compared with naïve mice (Fig. 4*C*).


A
Blood
Naive
Immune
2.7
2.3
Flank
0.39
2.2
CFSE
CD4
B
Blood
Flank
% CFSE+ CD4+
# CFSE+ CD4+
% CFSE+ CD8+
# CFSE+ CD8+
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C
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L. major
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E
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Immune αCD4
F
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Flank
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Naive Graft
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Graft: Naive Immune

Figure 4. *L. major*-specific T_{RM} cells enhance T cell recruitment following challenge with *L. major*. Splenocytes from *L. major* (A, B, C) or LCMV (D) immune mice were CFSE labeled to track them following transfer and were injected i.v. into either naïve or *L. major* immune mice (20 weeks post-infection) that were then challenged with *L. major* or PBS in the flank. Recruitment of cells to the challenge site was assessed 20 hrs later. (A) Representative flow plots of CFSE+ CD4+ T cells comparing the blood and the flank in naïve or immune mice challenged with *L. major*. (B) Frequency and number of transferred *L. major* immune CD4+ and CD8+ T cells in the blood and flank skin of naïve and immune mice 20 hours after challenge with *L. major*. (C) Frequency and number of transferred *L. major* immune CD4+ T cells 20 hours after challenge with *L. major* or injection of PBS in naïve or immune mice. (D) Frequency and number of transferred LCMV immune lymphocytes in the blood and flank skin of naïve and immune mice 20 hours after challenge with *L. major*. (E) *L. major* immune mice were treated with anti-CD4 mAb, and the frequency of CD4+ T cells in the skin of untreated or anti-CD4 treated immune mice is shown. (F, G) Naïve, *L. major* immune or *L. major* immune mice treated with anti-CD4 mAb (F) or anti-CXCR3 mAb (G) received CFSE-labeled splenocytes from immune mice, and were then infected in the flank with *L. major*. After 20 hr the frequency and number of the transferred cells in the blood and flank was determined. (H,I) Flank skin from naïve or immune mice was grafted onto the flank of naïve mice. After 7 days the recipient mice were injected intravenously (i.v.) with CFSE-labeled immune cells, each graft was infected with *L. major* and 20 hrs later cells isolated from the grafts. (H) Representative flow plots of CFSE+ CD4+ T cells comparing the blood and naïve or immune skin grafts challenged with *L. major.* (I) Frequency and number of transferred *L. major* immune CD4+ T cells in naïve or immune skin grafts (cells were gated on live, CD45, CD90.2, CD4 cells). All data are from one representative experiment of two or more with 3 or more mice per experiment, and each bar represents the mean ($\pm$ SEM) of individual mice (A-G) or combined data from two experiments where each dot represents a mouse (H, I). Immune mice used in all these experiments were 14 to 20 weeks post-infection. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

While the activation of leishmania T_{RM} cells appeared to be antigen-specific, we hypothesized that after antigen recognition these T_{RM} cells could recruit any circulating memory T cell (Schenkel et al., 2013). Indeed, when we transferred LCMV immune T cells to naïve and *L. major* immune mice, we also observed increased recruitment of T cells to immune skin following challenge (Fig 4*D*). Thus, these results show that antigen-specific stimulation of immune skin can promote increased recruitment of circulating memory T cells non-specifically.

To determine if T cell recruitment was dependent on the CD4+ T cells in the immune skin we compared the recruitment of transferred memory cells to immune and CD4+ T cell-depleted immune mice. CD4+ T cells were depleted from both the recipient and donor cell populations, so we focused on the recruitment of CD8+ T cells for these studies. Following anti-CD4 mAb treatment, no CD4+ T cells could be identified in the skin of immune mice (Fig 4*E*), and the enhanced recruitment of circulating memory cells observed in *L. major* immune skin was absent in anti-CD4 mAb treated mice, suggesting that the enhanced recruitment was CD4+ T cell dependent (Fig 4*F*).

We next assessed the role of chemokines in promoting the T cell recruitment to challenge sites by treating immune mice with anti-CXCR3 mAb. The chemokine receptor CXCR3 interacts with CXCL9, CXCL10, and CXCL11, and anti-CXCR3 mAb blocks CXCL10 and CXCL11 signaling *in vitro* (Uppaluri et al., 2008). We transferred labeled *L. major*-immune cells to intact and anti-CXCR3 mAb treated immune mice, challenged in the flank skin, and compared the recruitment of T cells to the challenge site 20 hours later. The increase in recruitment to immune flank skin was completely blocked in anti-CXCR3 mAb treated mice, indicating that this chemokine axis indeed promotes the enhanced T cell recruitment seen in immune mice (Fig 4*G*).

Finally, to determine if skin-resident cells were sufficient to drive the increased recruitment of activated T cells to immune skin, we grafted naïve and immune flank skin in parallel onto opposite sides of naïve recipients that lack any *L. major* memory cells. We then transferred circulating *L. major* immune cells, challenged both the naïve and immune graft with *L. major*, and compared recruitment of the labeled cells 20 hours later.

We observed enhanced recruitment of circulating cells to the immune graft compared to the naïve graft, indicating that cells from the skin alone could indeed mediate this effect (Fig. 4*H*, Fig. 4*I*). Together, these data indicate that *L. major*-responsive CD4+ T cells in the skin promote the early recruitment of circulating effector cells, and thus have the potential to improve the outcome of a secondary infection.

Leishmania-specific T_{RM} cells provide increased protection against secondary challenge

Finally, we sought to determine if T_{RM} cells could contribute to the control of *L. major* infection in immune mice. To specifically assess the effects of T_{RM} cells in the absence of circulating memory T cells, we grafted both naïve and immune skin onto a naïve recipient and challenged both skin grafts. After two weeks, we compared the parasite burden in each graft and found no significant difference between naïve and immune grafts (Fig. 5*A*). Thus T_{RM} cells did not appear to provide a significant level of protection by themselves. To assess if T_{RM} cell driven recruitment of circulating cells was important, we grafted naïve mice with both naïve and immune skin as before, but in addition we adoptively transferred *L. major*-specific memory cells isolated from immune mice. As expected, the presence of circulating *L. major*-specific cells significantly reduced the parasite burden even in the naïve graft two weeks post infection (Fig. 5*A*). However, the parasite burden in the immune graft was further significantly decreased compared to the naïve graft, indicating that immune skin was indeed better protected.

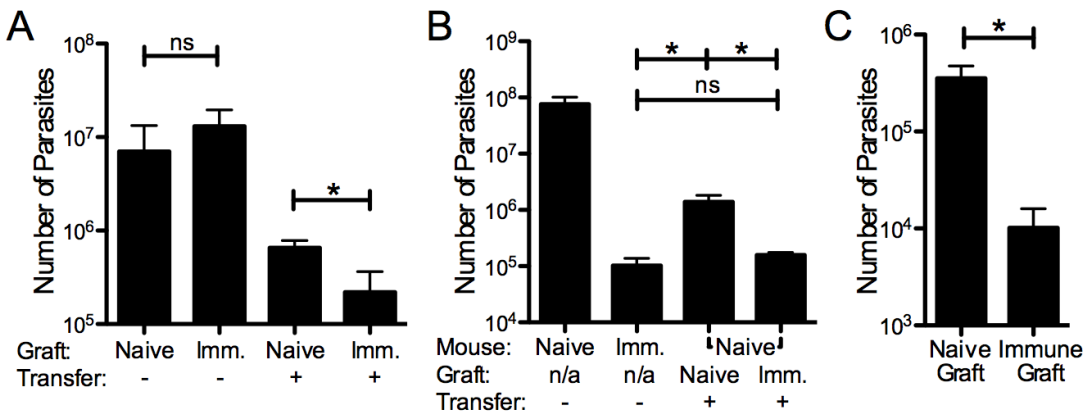

Figure 5. *L. major*-specific T_{RM} cells provide increased protection against secondary challenge. Flank skin from naïve or immune mice was grafted onto the flank of naïve mice. After 7 or 30 days, grafts from both the naïve and immune mice were challenged with *L. major* and two weeks later the grafts were isolated and parasite burdens measured. Some mice received 20 x 10^6 splenocytes (i.v.) from *L. major* immune mice at the time of challenge. (A) Naïve or immune skin grafts in the presence or absence of adoptively transferred *L. major* immune cells were challenged with *L. major* and the parasite burden was assessed at 2 weeks. Data shown are the mean (± SEM) parasite burden. (B) Intact naïve or immune mice, or naïve and immune skin grafted onto naïve mice were challenged with *L. major.* The mice with skin grafts also received *L. major* immune cells as above and the parasite burden was assessed at 2 weeks. The data shown are the mean (± SEM) of the parasite burden. (C) Naïve or immune skin was grafted onto naïve mice, and 30 days later mice received *L. major* immune cells as above and both grafts were challenged with *L. major.* The data shown are the mean (± SEM) of the parasite burden 2 weeks after *L. major* challenge. Data from A and B are representative of 2 experiments (n = 5 mice per group), and C is combined data from 2 experiments (n = 6 mice). Immune mice used in all these experiments were 14 to 20 weeks post-infection. *, $P < 0.05$.

To assess the level of protection achieved by transferring both circulating and skin-resident immune cells to naïve mice, we compared skin-grafted mice to intact immune mice 2 weeks after *L. major* challenge in the flank (Fig. 5*B*). As expected, intact immune mice were significantly protected compared to naïve mice, and were also better protected than naïve graft skin that had access to circulating cells alone. However, there was no difference between the level of protection observed in intact immune mice and

skin-grafted mice challenged in immune flank skin, suggesting that the presence of circulating and skin-resident T cells was sufficient for optimal protection.

Finally, to confirm that this protective phenotype was long lived, we grafted naïve and immune skin onto naïve mice allowed 30 days for the grafts to heal and revascularize. We then transferred in *L. major* immune cells, challenged each graft, and compared parasite burdens at 2 weeks. Even one month after grafting, the parasite burdens were significantly lower in immune skin, suggesting that *L. major*-specific T_{RM} cells were still mediating protective effects (Fig 5*C*). Together, these data support a model in which leishmania T_{RM} cells act as sentinels in the skin to rapidly recruit circulating memory cells and restrict parasite replication. Consequently, immunizations that do not induce leishmania T_{RM} cells may result in delayed recruitment of effector cells and impaired control of infection.

2.5 Discussion

We discovered that tissue-resident memory CD4+ T cells are present in the skin after resolution of leishmania infection. While resident memory T cells generated in response to acute viral infections have been extensively studied (Kim et al., 1999; Hogan et al., 2001a; Gebhardt et al., 2009; Wakim et al., 2010; Jiang et al., 2012; Schenkel et al., 2013; Teijaro et al., 2011; Iijima and Iwasaki, 2014), we identify for the first time a population of skin-resident CD4+ T cells that form in response to a chronic parasitic infection. These T_{RM} cells persist in the absence of circulating leishmania-specific T cells, are retained long after resolution of disease, produce IFNγ, and enhance the recruitment of circulating memory cells to the site of leishmania challenge. This rapid

mobilization of pathogen specific T cells significantly reduced the parasite burden, indicating that CD4+ T_{RM} cells contribute to protective immunity to leishmania.

While several subsets of leishmania-specific T cells are present in mice that have resolved a primary infection, adoptive transfer of these cells into naïve mice never recapitulates the level of protection seen in intact immune mice. Such results could stem from an inability to adoptively transfer adequate numbers of leishmania-specific T cells, however our current results documenting the ability of skin-resident leishmania memory cells to rapidly recruit circulating effector cells to the site of infection support an alternative hypothesis: that the absence of T_{RM} cells in recipient mice is responsible for the lower level of observed protection. Nevertheless, our results also show that these T_{RM} cells are insufficient to mediate protection alone, and require a circulating pool of leishmania-specific CD4+ T cells. These circulating cells have been well-characterized, and include effector ($CD62L^{lo}$, $IL\text{-}7R^{lo}$), effector memory ($CD62L^{lo}$, $IL\text{-}7R^{hi}$), or central memory ($CD62L^{hi}$, $IL\text{-}7R^{hi}$) $CD4^{+}$ T cells (Colpitts et al., 2009; Colpitts and Scott, 2010). Most recently, the circulating leishmania-specific T cells have been further characterized, and the most protective cells were shown to be Ly6C+ CD4+ effector T cells (Peters et al., 2014). Taken together, our data indicate that an optimal vaccine would need to generate both circulating protective CD4+ T cells and T_{RM} cells.

An important attribute of the leishmania-specific CD4+ T_{RM} cells is their persistence in the absence of chronic parasites. Specifically, leishmania-specific skin-resident memory T cells transferred by skin graft to uninfected mice persist for several weeks. This is in contrast to effector cells, which depend on low numbers of persisting parasites to be maintained (Belkaid et al., 2002a), and is most likely why optimal protection requires

chronic infection (Uzonna et al., 2001; Belkaid et al., 2002a; Sacks, 2014; Mendez et al., 2004). Nevertheless, some protection can be maintained in the absence of persisting parasites, which we previously attributed to the parasite-independent maintenance of central memory T cells (Zaph et al., 2004). Our discovery that CD4+ T_{RM} cells are maintained in skin in the absence of persistent parasites now identifies another parasite-independent memory T cell population that is an ideal target for a T cell vaccine.

The CD4+ T_{RM} cells that we identified in leishmania-infected mice appear to function by recruiting circulating effector cells. Transcriptional profiling of immune skin challenged with *L. major* showed increased expression of many IFNγ/STAT1 dependent genes, including the IFNγ inducible chemokines CXCL9 and CXCL10. By blocking the receptor for these chemokines CXCR3, we demonstrate that chemokine receptor signaling is important for the enhanced recruitment to challenge sites in immune mice. Although a similar role for CD8+ T_{RM} cells has been described (Schenkel et al., 2013; Ariotti et al., 2014), other studies suggest that CD8+ T_{RM} cells can control viruses by themselves, even when the recruitment of circulating cells is suppressed by treatment of mice with FTY-720 (Hofmann and Pircher, 2011; Jiang et al., 2012). This was not the case in leishmania infection, which might be due to inherent differences between CD4+ and CD8+ T_{RM} cells, or more likely might be related to the nature of the pathogen. Unlike acute viral infections that are quickly controlled even in naïve hosts, leishmaniasis is a chronic disease that takes months to heal, suggesting that rapid control of secondary challenges may require a particularly strong immune response. Thus, while leishmania-specific T_{RM} cells are an important component in the protection seen in immune mice, they also require the presence of circulating immune cells to promote protection.

Multiple strategies have been applied to leishmania vaccine development, including the use of different live or killed parasites, various adjuvants, and a multitude of DNA constructs and protein subunits (Coler and Reed, 2005; Kedzierski et al., 2006; Dunning, 2009). While some of these have provided a degree of protection (Palatnik-de-Sousa, 2012), there still is no human vaccine against leishmania (Noazin et al., 2008), and none of the experimental vaccines induce the level of protection seen in immune mice. Our results provide a new model for how leishmanial infections generate resistance to reinfection, one in which both circulating and tissue-resident cells work together to provide robust protective immunity. Taking this into account, new approaches to the development of a vaccine will need to consider not only the generation of circulating memory T cells, but also how best to generate a population of T_{RM} cells, which may be the missing link in the quest for a successful leishmania vaccine.

CHAPTER 3: Skin Resident CD4+ T cells Protect Against *Leishmania major* by Recruiting and Activating Inflammatory Monocytes

3.1 Abstract

Tissue-resident memory T cells are required for establishing protective immunity against a variety of different pathogens, although the mechanisms mediating protection by CD4+ resident memory T cells are still being defined. In this study we addressed this issue with a population of protective skin-resident, IFNγ-producing CD4+ memory T cells generated following *Leishmania major* infection. We previously found that resident memory T cells recruit circulating effector T cells to enhance immunity. Here we show that resident memory CD4+ T cells mediate the delayed-hypersensitivity response observed in immune mice and provide protection without circulating T cells. This

protection occurs rapidly after challenge, and requires the recruitment and activation of inflammatory monocytes, which limit parasites by production of both reactive oxygen species and nitric oxide. Overall, these data highlight a novel role for tissue-resident memory cells in recruiting and activating inflammatory monocytes, and underscore the central role that skin-resident T cells play in immunity to cutaneous leishmaniasis.

3.2 Introduction

Tissue-resident memory T cells (T_{RM}) are critical mediators of immunity against a number of different infections in a variety of different tissues (Jiang et al., 2012; Gebhardt et al., 2009; Iijima and Iwasaki, 2014; Laidlaw et al., 2014; Teijaro et al., 2011; Schenkel et al., 2014a; Masopust et al., 2010; Mackay et al., 2012; Cauley et al., 2002; Wakim et al., 2012; Sakai et al., 2014). Because they are typically located at barrier surfaces and therefore occupy the initial sites of infection, T_{RM} cells are poised to provide rapid protection. CD8+ T_{RM} cells are the best defined tissue-resident T cells, and mediate protection through direct cytotoxicity (Kim et al., 1999; Masopust et al., 2006; Steinbach et al., 2016), production of cytokines (Jiang et al., 2012; Schenkel et al., 2013), maturation of local innate cells (Schenkel et al., 2014a), triggering of tissue-wide antiviral signaling (Ariotti et al., 2014), and/or the recruitment of additional lymphocytes to the site of infection (Schenkel et al., 2013). CD4+ T_{RM} cells remain relatively uncharacterized, although they have been described in the lung, vaginal mucosa, and skin (Iijima and Iwasaki, 2014; Teijaro et al., 2011; Laidlaw et al., 2014; Collins et al., 2016). We recently demonstrated that skin-resident CD4+ T cells play a critical role in immunity to cutaneous leishmaniasis (Glennie et al., 2015), however the various mechanisms by which CD4+ T_{RM} cells mediate protection in the skin remain ill-defined.

Human cutaneous leishmaniasis encompasses a spectrum of diseases caused by the intracellular protozoan parasites. Murine models that mimic aspects of the human disease have proven invaluable for understanding the mechanisms mediating susceptibility and resistance (Scott and Novais, 2016). For example, similar to some forms of human cutaneous leishmaniasis, C57BL/6 mice infected with *Leishmania major* develop lesions that heal over several weeks, and once resolved the mice exhibit immunity to reinfection (Scott and Novais, 2016). Studies in this model have shown that in a primary leishmania infection, innate cells including neutrophils, monocytes, and dendritic cells are rapidly recruited to the site of challenge (Peters et al., 2008; Goncalves et al., 2011; Ng et al., 2008; Ribeiro-gomes et al., 2012). These cells have the potential to restrict parasite infection (Strauss-Ayali et al., 2007; Goncalves et al., 2011; Olekhnovitch et al., 2014; Novais et al., 2009), but they can also be co-opted by the parasites to evade immune detection or suppress the immune response (Peters et al., 2008; Afonso et al., 2008; van Zandbergen et al., 2004). Conversely, in a secondary infection, the recruitment of pre-existing circulating effector CD4+ Th1 cells leads to the rapid control of the parasites (Peters et al., 2014; Zaph et al., 2004), and CD4+ T_{RM} cells contribute by promoting the recruitment of these effector T cells to the site of infection (Glennie et al., 2015). However, given their location at the site of a challenge infection and their rapid production of IFNγ, it might be expected that CD4+ T_{RM} cells may also provide some level of rapid protection that is independent of additional T cell recruitment from the blood.

Here we show that CD4+ T_{RM} cells mediate control of the parasite burden within the first three days of infection, which correlates with a strong delayed-type hypersensitivity (DTH) response, the hallmark of immunity in murine and human leishmaniasis. While

IFNγ produced by T_{RM} cells might be expected to activate resident macrophages in the skin and limit the parasite burden, surprisingly we found that protection by CD4+ T_{RM} cells required the recruitment of inflammatory monocytes that subsequently controlled the parasites by the induction of both reactive oxygen species (ROS) and inducible nitric oxide synthase (iNOS). Importantly, we found that T_{RM} cells provided protection independently of circulating CD4+ T cells, emphasizing the importance of generating T_{RM} cells for optimal immunity to leishmaniasis.

3.3 Materials and Methods

Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee, University of Pennsylvania Animal Welfare Assurance Number 805186.

Mice

C57BL/6 mice were purchased from the National Cancer Institute (Fredericksburg, MD). CCR2-/- (B6.129S4-*Ccr2*^{tm1Ifc}/J), Phox-/- (B6.129S-*Cybb*^{tm1Din}/J), iNOS-/- (B6.129P2-*Nos2*^{tm1Lau}/J), and RAG-/- (B6.129S7-*Rag1*^{tm1Mom}/J) mice were purchased from The Jackson Laboratory. All mice were maintained in a specific pathogen-free environment at the University of Pennsylvania Animal Care Facility.

Parasites

L. major (Friedlin) or dsRed+ *L. major* (Friedlin) parasites were grown in complete Schneider's insect medium (GIBCO) supplemented with 20% heat-inactivated FBS,

2mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 50 µg/mL G418 sulfate (Cellgro) (CSM). Metacyclic enriched promastigotes were used for infection (Späth and Beverley, 2001). Mice were infected with 2 x 10^6 *L. major* or 10^3 *L. major* intradermally in the ear or flank skin as noted.

Antibodies and treatments

For flow cytometry analysis αCD45 APC-eF780, αCD45.2 FITC, αCD45.1PE-Cy7, αCD90.2 BV605, αCD11b BV650, αCD4 PE TexasRed, αCD8b PerCp/Cy5.5, αLy6C AF700, αLy6G PacBlue, αMerTK APC, αCD64 PE-Cy7, αCD11c FITC, αMHCII APC, αAF488 iNOS were incubated with single cell suspensions 30 minutes at 4°C and read on LSR Fortessa. For ROS stain, 2ng/mL dihydrorhodamine 123 (DHR, Cayman Chemical) was added directly ex vivo, then incubated 30 minutes at 37°C for 30 minutes. For *in vivo* blockade/depletion 250 µg of αCD4 (GK1.5), αCD8 (53-6.72), αCXCR3 (CXCR3-173), 500 µg of αGR1 (RB6-8C5), αLy6G (1A8) (BioXcell), or 1 mg/kg FTY-720 (Cayman Chemical) were given i.p. one day prior to *L. major* challenge.

Skin Preparation

For ear preparation, dorsal and ventral cutaneous layers of the ear were separated and incubated in RPMI (Gibco) with 250 µg/mL Liberase TL (Roche) for 90 minutes at 37°C in 5% CO_2. Cells from the skin were then dissociated using a 40 µm cell strainer (BD Pharmingen) and resuspended in complete RPMI media (cRPMI) containing 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 55 µM 2-Mercaptoethanol. For flank skin preparation, a section of skin was harvested from the flank following hair removal with an electric trimmer equipped with a two-hole precision blade (Wahl). Skin sections were then minced with a sterile scalpel blade into ~2mm sections, and incubated in

RPMI containing 1 mg each of type III and type IV collagenase (Worthington) for 120 minutes with vortexing every 30 minutes. The resulting solution was passed through a 40 µm cell strainer and resuspended in cRPMI. Bone marrow derived dendritic cells for restimulations were generated by culturing C57BL/6 bone marrow in GM-CSF supplemented cRPMI for 7-11 days. BMDCs were then harvested and infected 5-8 hours with stationary phase *L. major* at a ratio of 10:1 in the presence of 1 µg/ml CpG and LPS. Infected BMDCs were incubated at a ratio of 1:5 with 10^6 skin cells in 24 well plates for 12-16 hours. Cells were incubated for the last 4 hours with 5 µg/ml BFA (eBioscience), stained for IFNγ, and analyzed by flow cytometry.

Parasite quantification

Parasite burden from ear and flank skin was calculated by serial 2-fold dilution in 96-well plates of CSM and incubated at 26°C. The number of viable parasites was calculated from the highest dilution at which parasites were observed 7 days into culture. For qPCR, single cell suspensions from infected tissue were diluted in RLT lysis buffer, then RNA was isolated using the RNeasy Plus kit (Qiagen). RNA was converted to cDNA using the High Capacity RNA to cDNA kit (Applied Biosciences), then the Power SYBR green PCR mater mix (Applied Biosciences) was used to quantify parasite ssRNA on the ViiA7 qPCR machine (Applied Biosciences) with primers F: 5'-TACTGGGGCGTCAGAG-3' and R: 5'-GGGTGTCATCGTTTGC-3'. Cytospins were prepared at 1000 RPM (Shandon Cytospin3) and imaged by light microscopy at 40X magnification (Nikon E600).

Skin grafts

Skin grafts were performed as previously described (Glennie et al., 2015). Briefly, donor

skin was prepared under sterile conditions from naïve and immune mouse flank skin by shaving, depilating, cleaning with chlorhexidine (Vetoquinol), then excising the skin using sterile 8mm biopsy punches (Miltex). Grafts were placed onto a fresh graft bed prepared by excising skin using a 6mm biopsy punch. All mice were anesthetized, received analgesics, and were monitored post-operatively as previously described. In challenge experiments, graft skin was injected intradermally with 2×10^6 metacyclic *L. major* 14-20 days after grafting.

Parabiosis

Congenically disparate mice were co-housed 2 weeks prior to surgery. After induction of anesthesia with isoflorane, each received 0.1mg/kg buprenorphine subcutaneously (s.q.) as preemptive analgesia. The surgical site was shaved and aseptically prepared with chlorhexidine scrub. A longitudinal skin incision was made on the mirroring side in each mouse starting at 0.5 cm above the elbow and ending 0.5 cm below the knee joint. The left elbow and knee of one animal were attached to the right elbow and knee of the other parabiont with a 3-0 ethilon suture (Ethicon) around each joint beneath the skin in a manner loose enough to not disrupt circulation to the distal limb. The dorsal and ventral skin edges created by the flank incision from one mouse were sutured to the respective skin edges of the second mouse using a continuous absorbable 5-0 vicryl suture patter (Ethicon). Suture glue (Abbott laboratories) was used to approximate skin edges. 0.5 ml of 0.9% NaCl was administered s.q. to each mouse to prevent dehydration in the immediate post-operative recovery period, and mice were monitored twice daily for the first 48 hrs post-operatively, then observed daily for signs of surgical site complications, pain, or discomfort. In challenge experiments, ears were infected with 2×10^6 metacyclic *L. major* by intradermal injection14-20 days after surgery.

Statistical analysis

Statistical analysis was performed with the Student's T test (two-sided t-test, paired or unpaired where applicable), ANOVA, or 2-way ANOVA in Prism software (GraphPad).

3.4 Results

L. major immune mice are protected within 72 hours of challenge in a CD4+ T_{RM} cell dependent manner

In experimental models of cutaneous leishmaniasis, protection to a challenge infection is often assessed after several weeks, when a large difference in parasite number is evident between naïve and immune mice. This approach also allows for the assessment of protection mediated not only by circulating effector T cells, but also by central memory T cells that are delayed in their protective response (Zaph et al., 2004). However, the identification of T_{RM} cells and their occupation of the skin led us to hypothesize that they might contribute to immune protection very early after challenge. To test this, we challenged naïve and leishmania-immune mice in the ear with *L. major*, and assessed the immune response during the first 72 hrs of infection. For these studies, immune mice were infected with *L. major* in the contralateral ear at least 12 weeks earlier, and had resolved their primary lesion.

One of the hallmarks of immunity to leishmaniasis is the presence of a DTH response, and a positive reaction indicates that an individual has generated a type 1 immune response. As expected, immune mice developed a DTH response, represented by an increase in ear swelling within 24-72 hrs after challenge, while naïve mice did not (Fig. 1*A*). In order to evaluate whether the presence of this DTH reaction was associated with control of the challenge inoculum, we assessed the parasite burden by performing three

different assays: limiting dilution, qPCR for parasite ribosomal RNA, and analysis of the frequency of cells infected with dsRed expressing parasites by flow cytometry. We found that the number of parasites was consistently decreased 2-4 fold in immune mice at 72 hrs, as measured by limiting dilution and qPCR (Fig. 1*B*, 1*C*), and that the frequency of infected cells was significantly decreased by flow cytometry (Fig. 1*D*). These results demonstrate that as early as 72 hrs after challenge, mice that have resolved a previous *L. major* infection can mount an immune response that is effective at controlling the parasites.

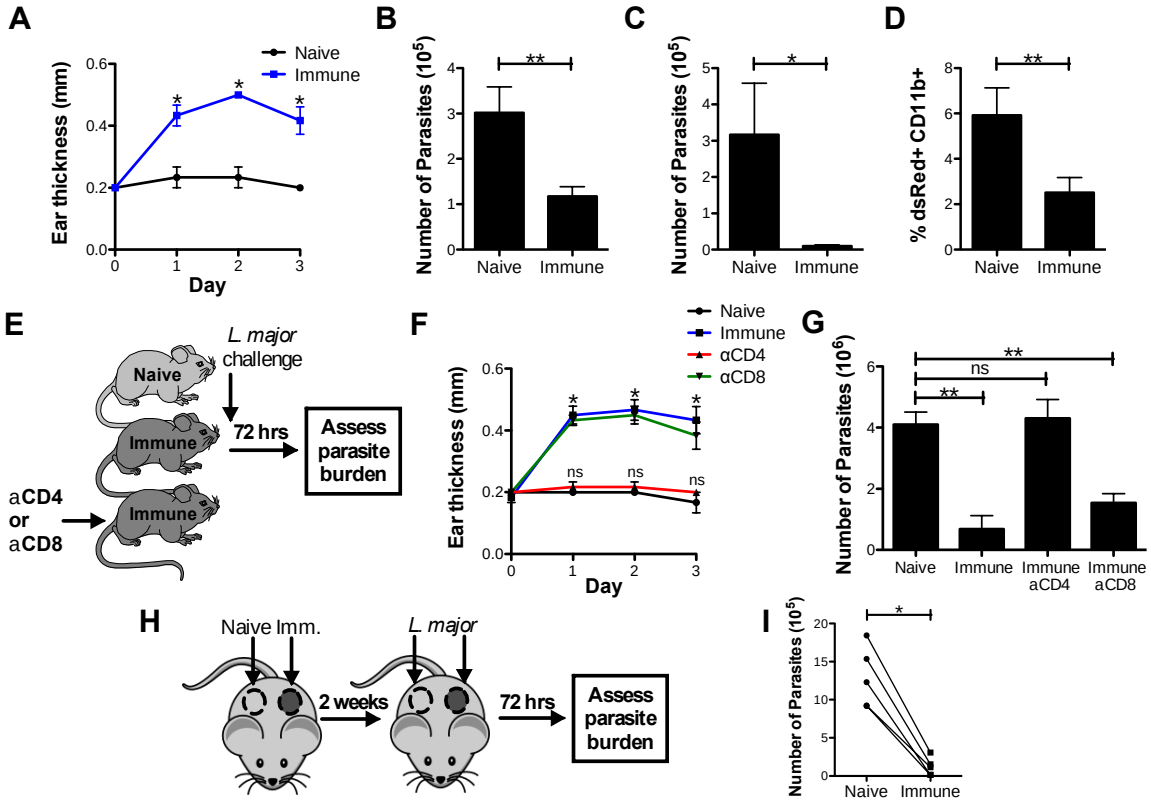

Figure 1. *L. major* immune mice are protected within 72 hrs of challenge in a CD4+ T_{RM} dependent manner. A) Naïve and immune mice were challenged intradermally in the ear with $2x10^6$ dsRed *L. major* and DTH response was monitored over the course of 3 days. (B-D) Representative parasite burden in naïve and immune mice 72 hrs after challenge as measured by limiting dilution (B), quantitative PCR (C), or flow cytometry (D). Representative data shown are from one experiment representative of eight (n

= 3-4 mice per group). E) Naïve, immune, and immune mice depleted of CD4+ or CD8+ T cells were challenged intradermally in the ear with $2x10^6$ dsRed *L. major*. F) DTH response was monitored over the course of 3 days. G) Parasite burden was determined at 72 hrs. Data shown are from one experiment representative of two (n = 3 mice per group). H) Naïve and immune flank skin were grafted side-by-side onto naïve recipients, then each graft was challenged intradermally with $2x10^6$ dsRed *L. major*. I) Parasite burden was determined at 72 hrs. Data shown are from one experiment representative of four (n = 5 mice per group). $P < 0.05$ = *; $P < 0.01$ = **; $P < 0.001$ = ***.

To determine if the DTH in leishmaniasis was dependent on either CD4+ or CD8+ T cells, we individually depleted each subset in immune mice before challenge with *L. major*, and then monitored the DTH response and parasite burden over 72 hrs (Fig. 1*E*). αCD4 treatment, which depletes both circulating and tissue-resident CD4+ cells in our hands (Fig. S1), completely ablated the DTH response (Fig. 1*F*), while effective CD8 depletion (Fig. S1) did not, suggesting that CD4+ cells are the critical drivers of this early inflammation. Importantly, CD4+ cells, but not CD8+ T cells, were also required for the decrease in parasites at 72 hrs (Fig. 1*G*).

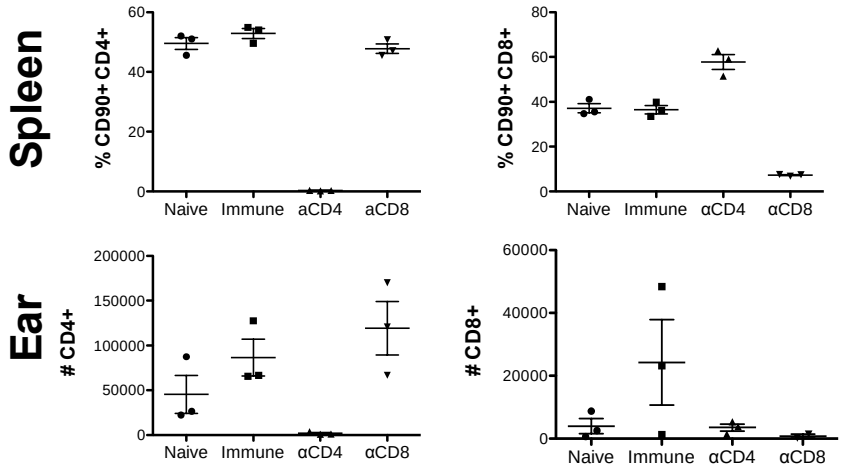

Figure S1. Efficacy of CD4 and CD8 depletions. Frequency or number of CD4+ and CD8+ cells in the spleen and challenged ear 72 hours after infection of CD4 and CD8 depleted immune mice are shown.

We next wanted to test if T_{RM} cells were mediating the early control of the parasites. To do so, we grafted naïve and immune skin side-by-side onto the flanks of naïve recipient mice, challenged each graft, and measured the parasite burden three days later (Fig. 1H). As the graft recipients contain only naïve T cells, this approach enabled us to specifically assess the protection mediated by T_{RM} cells, which we have previously shown to remain in the grafted tissue (Glennie et al., 2015). In all cases, the immune grafts had significantly fewer parasites than their naïve counterparts at 72 hrs (Fig. 1I). Taken together, these results indicate that CD4+ T_{RM} cells mediate parasite protection in immune skin at 72 hrs in a process that is independent of circulating CD4+ and CD8+ T cells.

Rapid protection in immune mice is associated with recruitment of inflammatory monocytes

To gain further insight into how this rapid protection is mediated, we analyzed the cells recruited to the skin of naïve and immune mice 72 hrs after challenge. We compared the numbers of CD90.2+ T cells, Ly6G+ neutrophils, Ly6C+ inflammatory monocytes, MerTK+ CD64+ macrophages, and CD11c+ MHCII+ dendritic cells in naïve and immune skin 72 hrs after infection (Fig 2*A*). As expected, we observed increased T cell recruitment to immune skin consistent with previous results (Glennie et al., 2015; Peters et al., 2014). However, a majority of the recruited cells were myeloid lineage cells, specifically inflammatory monocytes (Fig. 2*B*, 2*C*). We analyzed the activation status of these monocytes and found that they expressed high levels of MHCII, ROS, and iNOS. Further, both MHCII and iNOS expression were significantly increased in the monocytes recruited to immune skin compared with those recruited to naïve skin (Fig. 2*D*). Finally, using fluorescent parasites, we found that greater than 70% of the infected cells in the

skin of immune mice were inflammatory monocytes (Fig. 2*E*). Notably, these infected cells contained fewer parasites per cell when compared with monocytes in naïve skin, as demonstrated by the lower MFI of dsRed (Fig. 2F, 2G), and when counted in cytospins (Fig. 2*H*). These data show that monocytes are highly recruited to immune skin where they are more likely to be infected than other cell types, have a more activated phenotype, and contain fewer parasites per infected cell. These results suggest that inflammatory monocytes recruited by T_{RM} cells might be better able to kill parasites, and therefore we next investigated whether they were required for parasite control and if so how they mediated protection.

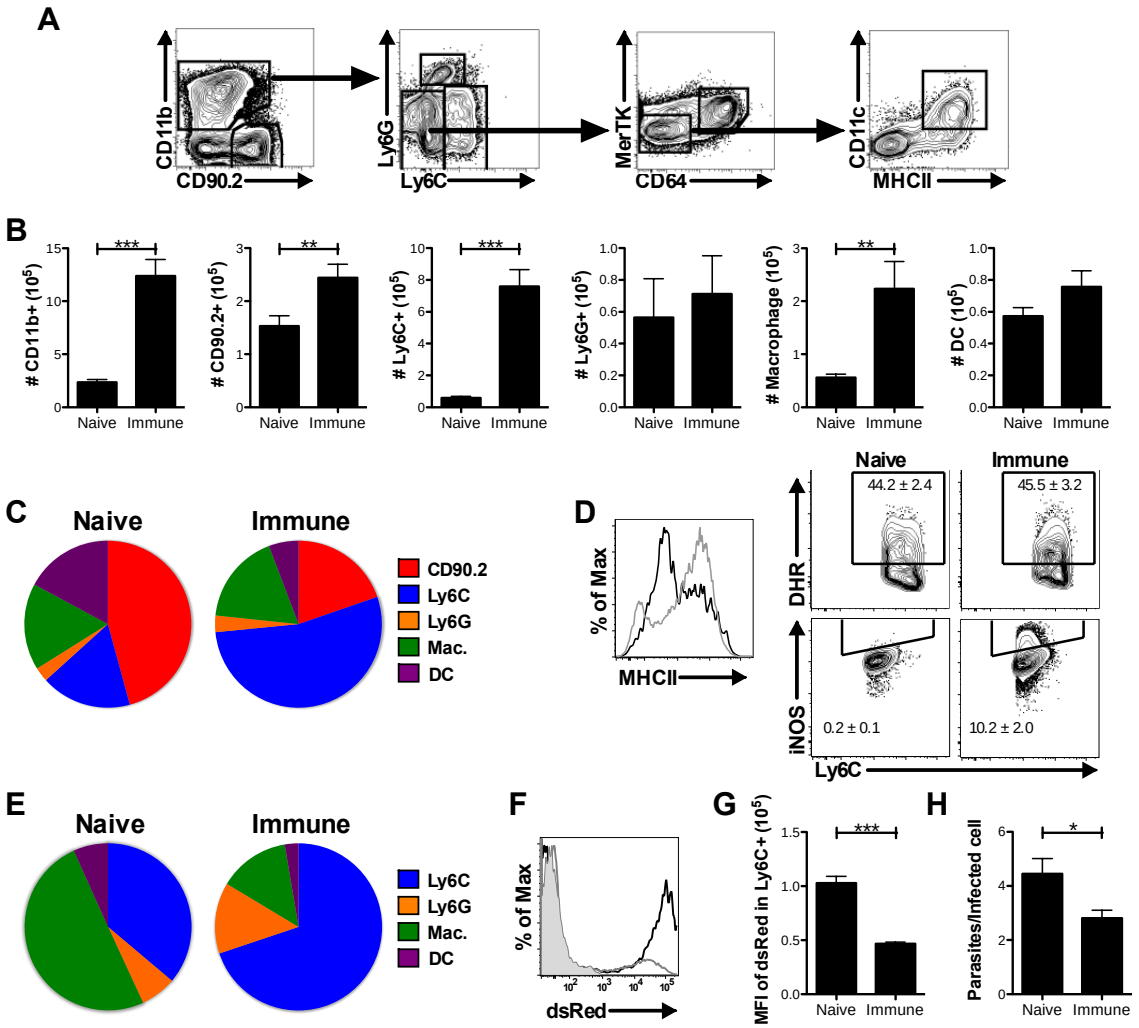

Figure 2. Rapid protection in *L. major* immune mice is associated with recruitment of inflammatory monocytes. A) Gating strategy for distinguishing myeloid cell populations. B) Numbers of T cells and myeloid cell populations in ear skin 72 hrs after challenge. C) Proportions of total CD45+ population in naïve and immune ears 72 hrs after challenge. Data shown are combined from six experiments (n = 18 mice per group). D) Representative histogram or contour plots showing frequency of MHCII+, DHR+, and iNOS+ monocytes in naïve (black line) and immune (gray line) mice 72 hrs after challenge. Data shown are from one experiment of two (n = 3 mice per group). E) The proportion of infected CD11b+ dsRed+ cells comprised of each cell type 72 hrs after challenge. Data shown are combined from six experiments (n = 18 mice per group) F) Representative plot of dsRed expression in CD11b+ cells from uninfected (gray fill), naïve (black line), or immune (gray line) mice. G) Quantification of dsRed MFI in Ly6C$^+$ cells from naïve or immune skin 72 hrs after challenge. Data shown are combined from two experiments (n = 8 mice per group). H) Number of parasites per infected cell counted from 50 cells per

cytospin slide. Data shown are combined from two experiments (n = 5-6 mice per group). $P < 0.05$ = *; $P < 0.01$ = **; $P < 0.001$ = ***.

Early protection is dependent on inflammatory monocytes

To assess the role of inflammatory monocytes in the early protection of immune mice, we used a pair of depleting antibodies that target either Ly6G+ and Ly6C+ cells (and thus deplete both neutrophils and monocytes) or Ly6G+ cells alone (and thus deplete only neutrophils) (Fig. 3*A*, Fig. S2). Depletion of neutrophils alone had no effect on the DTH response in immune mice, but depletion of both neutrophils and monocytes dramatically reduced the early inflammatory response (Fig. 3*B*). Importantly, the protection observed in immune mice was also completely ablated by the depletion of monocytes and neutrophils, while depleting neutrophils alone did not have a significant effect (Fig. 3*C*). Because activated CD4+ cells can also express Ly6C, we confirmed that RB6-8C5 treatment did not reduce the frequency of Ly6C+ CD4+ T cells in the spleen or ear after challenge (Fig. S3), or the frequency of leishmania-specific IFNγ+ skin-resident T cells (Fig. S4), which are intermediate for Ly6C expression (Fig. S5). Taken together, these data suggest that inflammatory monocytes are the critical mediators of early protection.

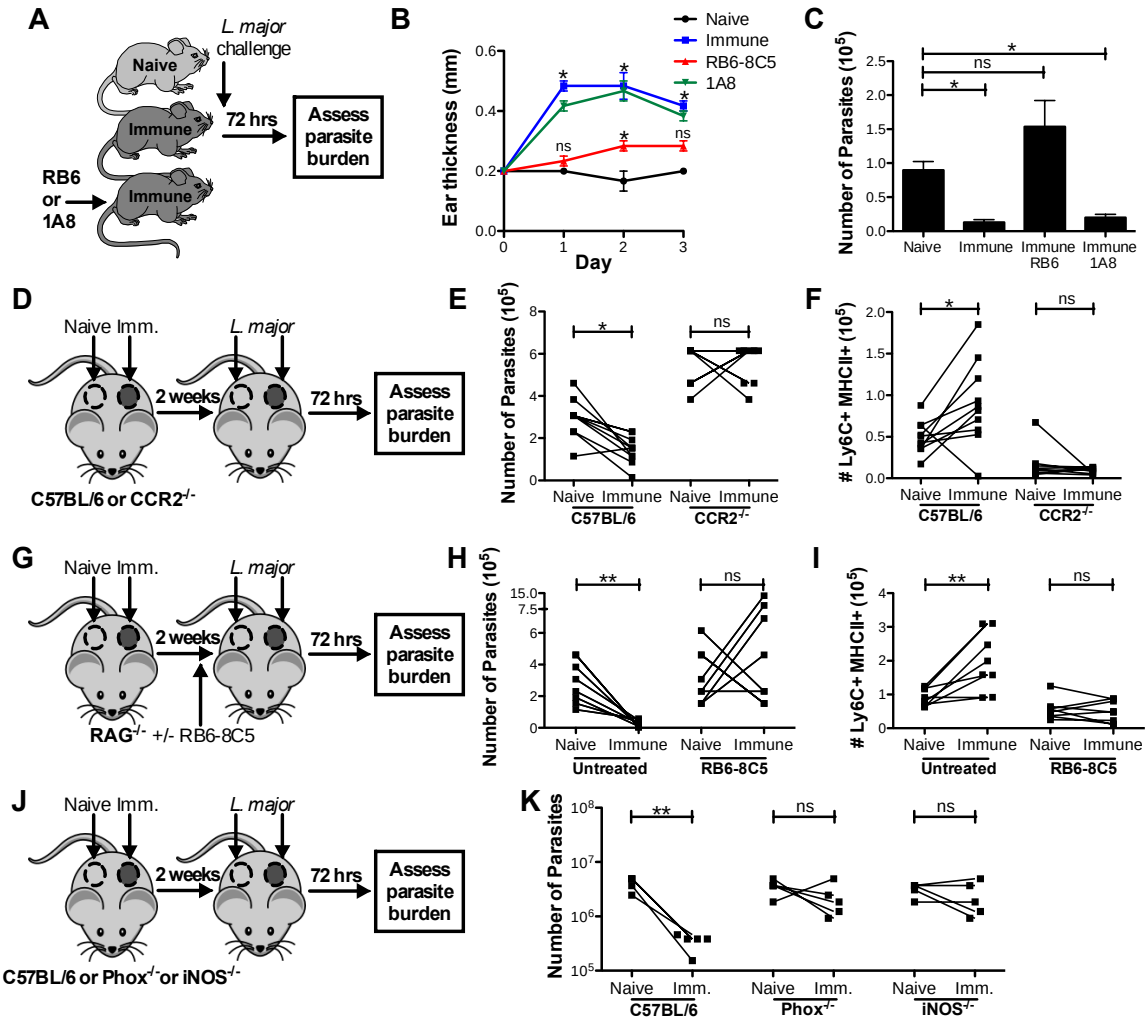

Figure 3. Early protection is dependent on inflammatory monocytes. A) Naïve, immune, and immune mice depleted with αGR1 clone RB6-8C5 (neutrophils and monocytes) or 1A8 (neutrophils only) were challenged intradermally in the ear with 2x10^6 dsRed *L. major*. B) DTH response was monitored over the course of 3 days. C) Parasite burden at 72 hrs was determined by limiting dilution. Data shown are from one experiment representative of two (n = 3 mice per group). D) Naïve and immune flank skin were grafted side-by-side onto WT or CCR2$^{-/-}$ naïve recipients, then each graft was challenged intradermally with 2x10^6 dsRed *L. major*. E, F) Parasite burden was determined in each graft by limiting dilution (E), and the number of activated Ly6C+ MHCII+ monocytes was determined at 72 hrs (F). Data shown are combined from two experiments (n = 9-10 mice per group). G) Naïve and immune flank skin were grafted side-by-side onto RAG-/- recipients. Half the mice were treated with 500μg αGR1 clone RB6-8C5 one day prior to challenge, then each graft was challenged with 2x10^6 dsRed *L. major*. H) Parasite burden was determined in each graft by limiting dilution at 72 hrs. I) The number of Ly6C+ MHCII+ cells in each graft was quantified. Data shown are combined

from two experiments (n = 4 mice per group). J) Naïve and immune flank skin were grafted side-by-side onto WT, PHOX-/-, or iNOS-/- naïve recipients, then each graft was challenged with $2x10^6$ dsRed *L. major*. K) Parasite burden was determined in each graft by limiting dilution at 72 hrs. Data shown are from one experiment representative of two (n = 5 mice per group). $P < 0.05$ = *; $P < 0.01$ = **; $P < 0.001$ = ***.

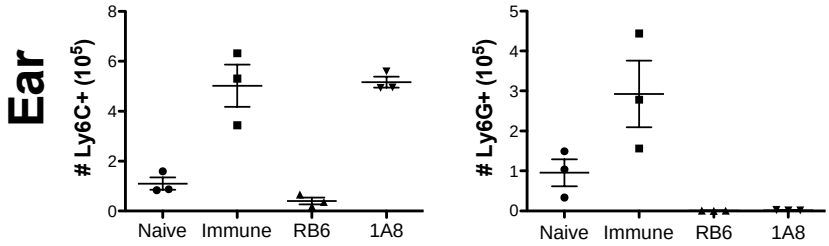

Figure S2. Efficacy of RB6-8C5 and 1A8 depletions. Number of Ly6C+ and Ly6G+ cells in the challenged ear 72 hours after infection of RB6-8C5 or 1A8 treated immune mice are shown.

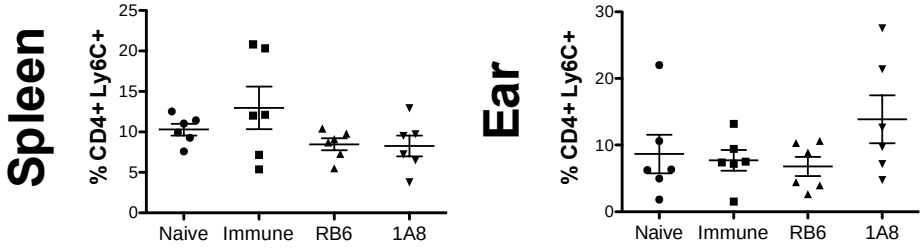

Figure S3. RB6-8C5 treatment does not ablate circulating Ly6C+ CD4+ T cells. Frequency of Ly6C+ CD4+ T cells in the spleen and challenged ear 72 hours after infection of RB6-8C5 or 1A8 treated immune mice are shown.

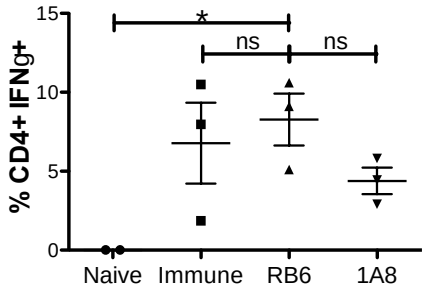

Figure S4. RB6-8C5 treatment does not deplete T_{RM} cells. The frequency of T_{RM} cells, as represented by IFNγ+ CD4+ T cells in the flank skin upon restimulation with *L. major* infected BMDCs, is shown for immune mice treated with 500μg RB6-8C5 or 1A8.

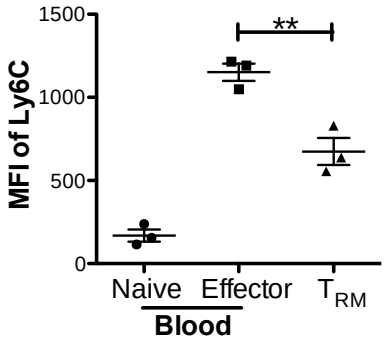

Figure S5. Ly6C expression is intermediate on T_{RM} cells. Comparison of Ly6C MFI on naïve or Ly6C+ effector cells from the blood and T_{RM} cells from the flank, as represented by cells that produced IFNγ in response to restimulation with *L. major* infected BMDCs.

To specifically address whether inflammatory monocyte recruitment is critical to early protection against *L. major*, we assessed the response to challenge in CCR2-/- mice, which contain monocytes that lack the ability to respond to CCL2 and CCL7 chemokine signaling and therefore cannot be efficiently recruited to sites of inflammation (Tsou et al., 2007; Boring et al., 1997). To do so, we grafted naïve and immune skin from WT mice onto the flanks of naïve WT or CCR2-/- recipients, challenged with *L. major*, and measured the parasite burden 72 hrs later (Fig. 3*D*). As previously observed, immune skin had significantly fewer parasites compared to naïve skin in WT recipient mice (Fig. 3*E*). In contrast, the reduction of parasites in immune skin was lost in CCR2-/- recipient

mice (Fig. 3*E*), and correlated with a loss of activated monocytes in the skin (Fig. 3*F*). Together, these results demonstrate that it is recruited CCR2+ monocytes, rather than resident myeloid cells, that are required for protection.

To further confirm that inflammatory monocytes were necessary for early protection, and that this protection could be conferred in the absence of circulating T cells, we grafted WT naïve and immune skin onto the flanks of RAG-/- recipient mice that lack T and B lymphocytes. Additionally, we treated some of the mice with αGR1 clone RB6-8C5 to deplete inflammatory monocytes and neutrophils as described above. We challenged each graft with *L. major* and measured the parasite burden 72 hrs later (Fig. 3*G*). As expected, immune grafts on RAG-/- mice contained significantly fewer parasites, demonstrating that the protection observed at 72 hrs was independent of circulating lymphocytes (Fig. 3*H*). Protection in immune skin was lost in mice treated with RB6-8C5 (Fig. 3*H*). When we quantified the number of Ly6C+ MHCII+ cells in each graft, we found a strong correlation with the level of protection (Fig. 3*I*). Taken together, these data further implicate inflammatory monocytes as the critical cell type required for early protection.

To gain further insight into the mechanism by which the inflammatory monocytes might control the parasites, we performed skin graft experiments in which we grafted naïve and immune skin onto the flank of 1) WT naïve mice, 2) Phox-/- mice in which monocytes lack the ability to produce ROS, or 3) iNOS-/- mice that have deficient NO production (Fig. 3*J*). Grafts were then challenged with *L. major*, and the parasite burden measured at 72 hrs. We found that the protection associated with immune skin was lost in both the

Phox-/- and iNOS-/- mice (Fig. 3*K*), suggesting that both ROS and NO from inflammatory monocytes are required for this early protection.

Early protection is not enhanced by circulating memory T cells

Although T_{RM} cell-mediated recruitment of inflammatory monocytes was sufficient to reduce the parasite burden at 72 hrs, we predicted that the presence of circulating leishmania-specific T cells might further enhance immunity, as they are also recruited early after challenge (Glennie et al., 2015, Peters et al. 2014). To examine the contribution of circulating T cells, we pretreated immune mice with either FTY-720, which prevents egress of T cells from tissues, or αCXCR3, which we previously demonstrated blocks the ability of T_{RM} cells to recruit effector T cells from circulation (Glennie et al., 2015) (Fig. 4*A*). Despite the expected decrease in the number T cells recruited to the challenge site (Fig. S6), neither treatment affected the DTH response (Fig. 4*B*), the decrease in parasite burden (Fig. 4*C*), or the recruitment of monocytes (Fig. 4*D*). Unexpectedly, this result demonstrates that DTH and early parasite control are not enhanced by T cells from circulation, and implies that CD4+ T_{RM} cells are solely responsible for mediating these responses.

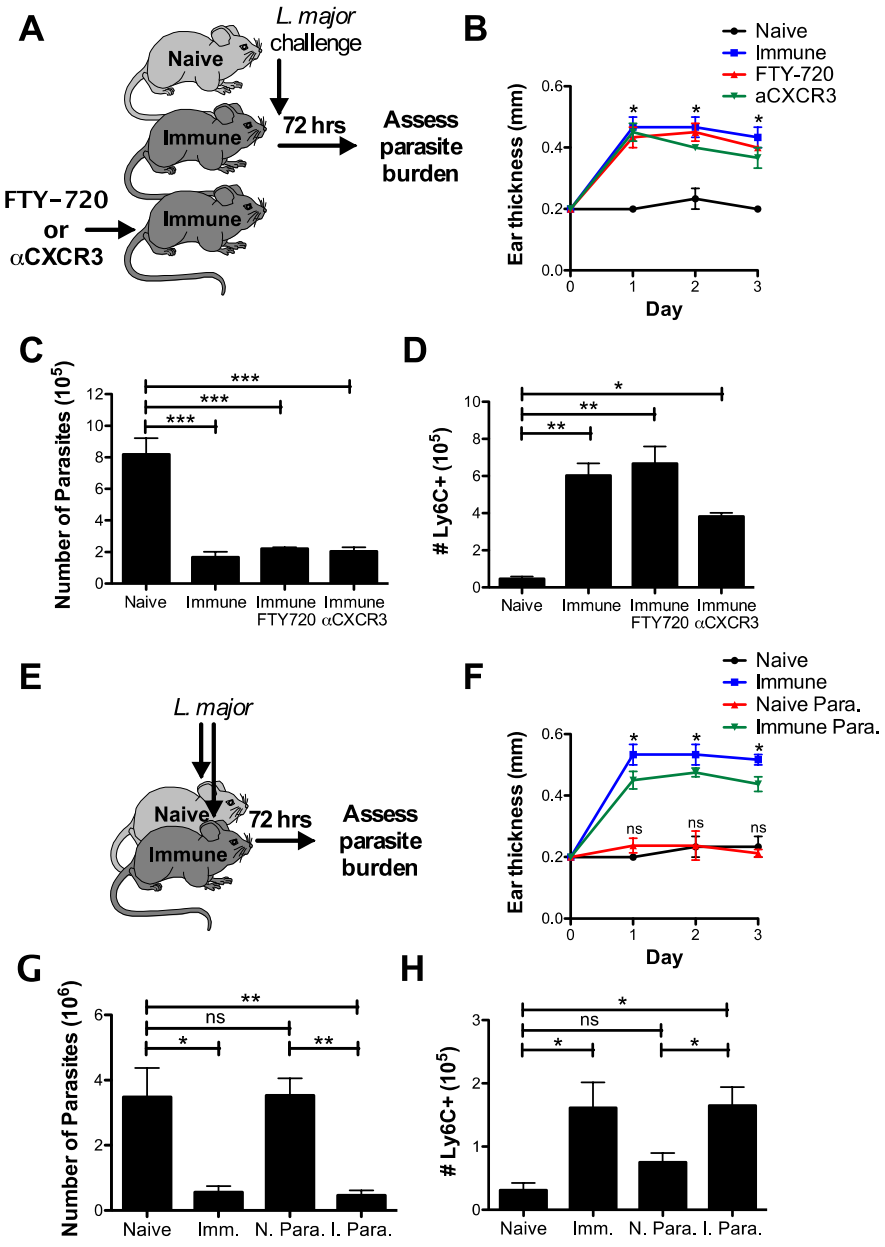

Figure 4. Early protection is not enhanced by circulating memory T cells. A) Naïve, immune, and immune mice treated with FTY-720 or αCXCR3 were challenged intradermally in the ear with $2x10^6$ dsRed *L. major*. B) DTH response was monitored over the course of 3 days. C, D) Parasite burden (C) and the number of Ly6C+ monocytes in the infection site (D) was determined at 72 hrs. Data shown are from one experiment representative of two (n = 3 mice per group). E) Naïve, immune, naïve parabiotic, and immune parabiotic mice were challenged intradermally in the ear with $2x10^6$ dsRed *L. major*. F) DTH

response was monitored over the course of 3 days. G, H) Parasite burden (G) and the number of Ly6C+ monocytes in the infection site (H) was determined at 72 hrs. Data shown are from one experiment representative of two (n = 3 mice per group). $P < 0.05$ = *; $P < 0.01$ = **; $P < 0.001$ = ***.

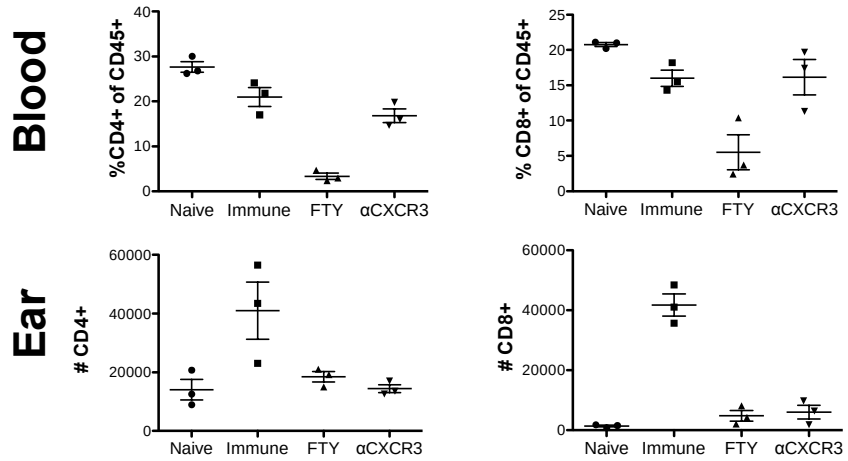

Figure S6. Efficacy of FTY-720 and αCXCR3 Blockade. Frequency or number of CD4+ and CD8+ cells in the blood and challenged ear 72 hours after infection of FTY-720 or αCXCR3 treated immune mice are shown.

To test if circulating T cells would provide protection in the absence of T_{RM} cells, we utilized a parabiotic model in which the circulations of naïve and immune mice were surgically joined, allowing circulating T cells to equilibrate between the two animals, while T_{RM} cells remained exclusively in the immune partner (Fig. 4*E*). Each parabiont was then challenged with *L. major* in the ear, and DTH and parasite number were measured 72 hrs later. As expected, immune parabionts had the same DTH response, monocyte recruitment, and parasite numbers as control immune mice (Fig. 4*F-H*). In contrast, naïve parabionts, despite having a full complement of circulating memory T cells, did not exhibit a DTH response, had defective monocyte recruitment, and lost the early protection observed in immune mice (Fig. 4*F-H*). These results show that circulating leishmania-specific T cells by themselves are unable to provide any

protection at this early time point, further demonstrating that T_{RM} cells are the critical subset for this rapid protection.

Circulating memory T cells are not required to control low dose *L. major* infection

In contrast to the parasite control observed at 72 hrs in immune mice, we previously found that when protection was assessed two weeks after challenge with 2×10^6 parasites, optimal parasite control depended upon both CD4+ T_{RM} cells and circulating effector T cells (Glennie et al. 2015). These results, in combination with our current findings, suggest that while T_{RM} cells may initially reduce the parasite number, the long-term consequences are limited in the absence of additional circulating T cells. However, since parasite dose can significantly influence what is required for protection, and the number of infective parasites transmitted by the sand fly is thought to be much lower than 2×10^6 parasites (Kimblin et al., 2008), we next tested whether T_{RM} cells might provide protection greater than 72 hrs after challenge if fewer parasites were present in the challenge inoculum.

First, we challenged naïve and immune mice with 10^3 parasites in the ear, measured the DTH, and assessed the composition of cells recruited to the challenge site at 72 hrs. Similar to our results with high dose challenge, immune mice had an increased DTH response (Fig. 5*A*), there was a large population of inflammatory monocytes infiltrating the lesions (Fig. 5*B*), and the monocytes had a more activated phenotype (Fig. 5*C*), though the magnitude of the overall response was lower.

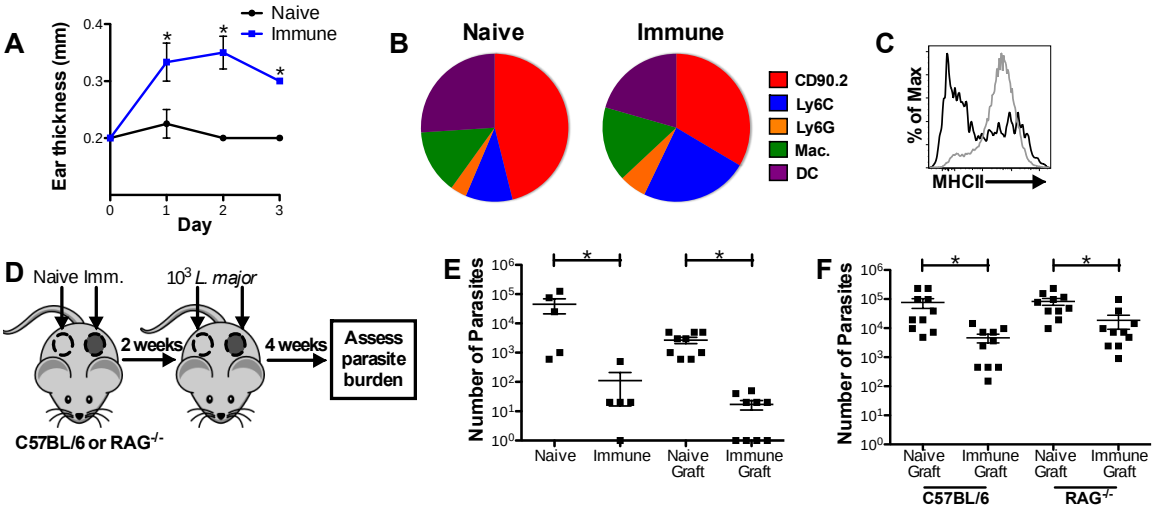

Figure 5. Circulating memory T cells are not required to control low dose *L. major* infection. A) Naïve and immune mice were challenged in the ear with 10^3 parasites and DTH was monitored over 72 hrs. B) Proportions of total CD45+ population in naïve and immune ears 72 hrs after low dose challenge. C) Representative histogram showing frequency of MHCII cells in naïve (black line) and immune (gray line) mice 72 hrs after low dose challenge. Data shown are from one experiment of two (n = 3 mice per group). D) Naïve and immune flank skin were grafted side-by-side onto naïve WT or RAG-/- mice, then each graft was challenged with 10^3 dsRed *L. major*. E) Parasite burden at 4 weeks was compared between intact naïve and immune mice as well as naïve and immune grafts on WT recipients. Data shown are from one experiment representative of two (n = 5 or 9 mice per group). F) Parasite burden at 4 weeks was compared between naïve and immune grafts on WT or RAG-/- recipients. Data shown are combined from two experiments (n = 5 mice per group). $P < 0.05$ = *; $P < 0.01$ = **; $P < 0.001$ = ***.

To test if T_{RM} cells could provide protection more than 72 hrs after challenge without circulating T cells, we challenged intact naïve mice, immune mice, naïve grafts, and immune grafts with 10^3 *L. major*, and measured parasite burdens 4 weeks later (Fig. 5*D*). As expected, intact immune mice were better protected than naïve mice against low dose *L. major* challenge (Fig. 5*E*). However, immune skin grafts also showed significantly better protection than their naïve counterparts, despite the absence of circulating leishmania-specific effector T cells (Fig. 5*E*). These results demonstrate that T_{RM} cells do not require previously activated circulating T cells to provide protection as

long as 4 weeks after low dose challenge. However, naïve leishmania-specific T cells would be expanded during the 4 weeks of infection, and could contribute to the protection we measured. Therefore, to test if T_{RM} cells could provide protection without any circulating lymphocytes, we grafted naïve and immune skin onto WT and RAG-/- recipient mice, challenged with 10^3 parasites, and measured the parasite burden 4 weeks later (Fig. 5*D*). Surprisingly, we found that the immune grafts showed significantly better control of the parasites in both WT and RAG-/- mice (Fig. 5*F*). While circulating effector T cells may have potential to contribute to long-term immunity, these results indicate that T_{RM} cells and innate cells alone are sufficient to provide a significant level of protection.

3.5 Discussion

We recently reported that T_{RM} cells provide optimal immunity against *L. major* infection by recruiting circulating leishmania-specific effector T cells to the site of infection (Glennie et al., 2015). We now identify an additional, novel function for leishmania-specific T_{RM} cells: to rapidly recruit and activate inflammatory monocytes at the site of infection, resulting in a significant reduction in the initial parasite burden. Further, we show that when the challenge inoculum is at a physiologically relevant dose, CD4+ T_{RM} and inflammatory monocytes exhibit significant control of the parasites, even when circulating leishmania-specific effector T cells are not present. Together, these results demonstrate that in addition to facilitating the recruitment of circulating effector T cells, CD4+ T_{RM} cells play a primary role in controlling parasites immediately after challenge, which not only indicates the importance of generating CD4+ T_{RM} cells in a vaccine, but also expands our understanding of the functions of CD4+ T_{RM} cells.

Our experiments have identified CD4+ T_{RM} cells as the critical cell subset required for both the DTH response and the immediate control of leishmania infection. The response is antigen specific as it is not induced by PBS injection, and is likely initiated via local antigen presentation (Khan et al., 2016). The identification of T_{RM} cells as required for DTH responses was unexpected, since the prevailing view was that circulating effector T cells mediated DTH. However, our results are similar to those that have been described in studies of contact-hypersensitivity, where T_{RM} cells mediated the inflammatory response independent of circulating T cells (Gaide et al., 2015). Since DTH responses can be elicited at sites distal to the initial site of infection, these results confirm that CD4+ T_{RM} are distributed and can function throughout the skin. Thus, our results extend those of others which have focused on the functions of CD4+ T_{RM} cells at the site of infection (Iijima and Iwasaki, 2014; Collins et al., 2016). Surprisingly, neither CD8+ T cells nor circulating effector T cells are required for the DTH response and the early control of the parasites, implicating CD4+ T_{RM} as the mediators of the initial inflammatory responses.

We found that inflammatory monocytes are rapidly recruited to the lesion site by T_{RM} cells, and are responsible for the observed protection. Inflammatory monocytes are important mediators of protection against many viral (Lim et al., 2011; Aldridge et al., 2009) bacterial (Serbina et al., 2003; Peters et al., 2001; Moyat et al., 2015), fungal (Hohl et al., 2009; John J. Osterholzer, Gwo-Hsiao Chen, Michal A. Olsewski, Jeffrey L. Curtis, Gary B. Huffnagle, 2014), and parasitic infections (Robben et al., 2005; Strauss-Ayali et al., 2007; León et al., 2007), and thus this mechanism of protection has the potential to influence a number of different immune responses. Inflammatory monocytes are known to be activated by memory T cells (Soudja et al., 2014), and restrict

leishmania infection in certain contexts (León et al., 2007; Strauss-Ayali et al., 2007; Goncalves et al., 2011), but their role in secondary leishmania infection and interaction with T_{RM} cells has not previously been appreciated. On the other hand, neutrophils did not appear to be required for early protection or the recruitment of inflammatory monocytes. This is in contrast to a primary infection where neutrophils may contribute to the recruitment of dendritic cells (Charmoy et al., 2010). This difference is most likely due to the presence of T_{RM} cells, which are sufficient to mediate phagocyte recruitment in secondary challenge. Indeed, CCL2 and CCL7 transcripts are both increased 12 hrs after challenge in immune mice (Glennie et al., 2015), and CCR2 signaling is required for the recruitment of inflammatory monocytes and subsequent protection.

ROS and NO from myeloid lineage cells have both been shown to have roles in controlling leishmania infections, though results vary based on the site of infection, the species of parasite involved, and whether the studies are done in mice or humans (Blos et al., 2003; Stenger et al., 1996; Goncalves et al., 2011; Liew et al., 1991; Carneiro et al., 2016; Novais et al., 2014). Nonetheless, it is clear in our model that both ROS and NO are required for full protection early after challenge of immune mice, and neither is sufficient alone. Thus, while most studies in mice have emphasized the central role of NO, it has become clear that ROS can contribute to protection not only in humans but also in the mouse. For example, following *L. major* infection Phox-/- mice can develop chronic lesions long after presumed cure (Blos et al., 2003). Understanding why ROS is required under certain conditions for control of leishmanial infections in mice is still not well understood, although we would speculate that at the early time point the levels of NO induced may be insufficient for parasite control, and ROS are required to boost the killing by the inflammatory monocytes.

Currently there is no human vaccine for leishmaniasis, which has been partially attributed to the inability to maintain sufficient circulating effector T cells following immunization (Mendez et al., 2004; Uzonna et al., 2001; Belkaid et al., 2002a). Thus, it is the presence of low numbers of parasites in immune mice that are believed to maintain maximal levels of responsive effector cells (Peters et al., 2014; Zaph et al., 2004; Belkaid et al., 2002a; Mendez et al., 2004). However, our studies indicate that T_{RM} cells can mediate protection alone, suggesting that these T cells should be targeted for a vaccine. Importantly, we found that they can survive in the absence of persistent parasites (Glennie et al., 2015), similar to central memory T cells (Zaph et al., 2004), and thus if generated in a vaccine may be maintained long-term. Thus, defining the requirements for the generation and maintenance of T_{RM} cells, as well as developing vaccination strategies that induce T_{RM}, are the next important steps in developing a vaccine for leishmaniasis.

As our understanding of tissue resident T cells grows, more functions have been attributed to T_{RM} cells. CD8+ T_{RM} can be directly cytotoxic (Kim et al., 1999; Masopust et al., 2006), and IFNγ from T_{RM} cells has been shown to drive recruitment of circulating T cells (Schenkel et al., 2013; Glennie et al., 2015). Transcriptional analyses have identified a core set of changes induced by T_{RM} activation that induce a tissue state of pathogen alert, capable of protecting against viral challenge non-specifically (Ariotti et al., 2014; Schenkel et al., 2014a). However, this is the first demonstration to our knowledge of T_{RM} cells orchestrating the innate response and classic DTH responses by recruiting inflammatory monocytes to the site of infection. This protective mechanism has the potential to be relevant for a number of different intracellular infections, as DTH responses are the hallmark of immunity against many infections and inflammatory

monocytes are potent killers of many pathogens (Shi and Pamer, 2011). Although circulating effector T cells are undoubtedly beneficial, in certain contexts the rapid response provided by a combination of CD4+ T_{RM} cells and inflammatory monocytes that lessen the initial pathogen burden may be critical in limiting the magnitude of the disease.

CHAPTER 4: Skin Resident CD4+ T Cells are Formed from Activated Effector Cells that enter Uninflamed Skin Within 2 Weeks of Infection

4.1 Abstract

A population of CD4+ tissue-resident memory T cells forms after cutaneous leishmania infection and appears to infiltrate throughout the skin, providing increased protection against challenge. In order to leverage these cells for therapeutic advantage, specifically in the context of a vaccine, we would like to understand how this population of cells forms, and particularly how these cells gain entry to uninflamed skin sites. Here we have examined the timing with which CD4+ T_{RM} cells are established, finding that they infiltrate uninflamed skin within 2 weeks of challenge, but can no longer enter once infection is resolved. However, reactivation of memory T cells appears to license them to enter uninflamed skin, and even boosts responses in immune mice. In a direct comparison, we identify T effector cells as superior to T memory cells at entering uninflamed skin, suggesting that these are the cells to target in a vaccine to generate T_{RM} cells.

4.2 Introduction

Tissue-resident T cells (T_{RM}) have proven to be critical mediators of protection against a number of different viral (Jiang et al., 2012; Mackay et al., 2012; Gebhardt et al., 2009; Laidlaw et al., 2014; Teijaro et al., 2011; Schenkel et al., 2013; Shin and Iwasaki, 2012), bacterial (Bergsbaken and Bevan, 2015; Sakai et al., 2014), and parasitic infections (Tse et al., 2013; Glennie et al., 2015). Once a tissue specific infection is resolved, T_{RM} cells are retained at the site of infection, and are thus capable of initiating a secondary response much more rapidly. While T_{RM} cell-derived protective responses are well studied at the site of infection, much less is known about T_{RM} cells at secondary sites distal to the initial site of challenge, though it is clear that T_{RM} cells infiltrate tissue broadly in at least some contexts (Masopust et al., 2001; Jiang et al., 2012; Glennie et al., 2015).

Leishmania major is an intracellular, protozoan pathogen that causes self-limiting skin lesions in humans and C57BL/6 mice. Similar to humans, immune mice that have resolved a primary infection are highly resistant to reinfection, and we have shown that this protection is at least partially dependent on a population of CD4+ T_{RM} cells that appear to distribute throughout the skin (Glennie et al., 2015, Glennie et al. 2017). Importantly, although chronic parasites are maintained at the primary site of challenge, parasites are not detected in distal skin sites, suggesting that T_{RM} generation and maintenance occurs independently of local inflammation.

Despite significant advances, the signals that drive the generation of T_{RM} cells in tissue remain somewhat undefined. Once T cells have entered the tissue, it is clear that tissue-specific signals can drive the subsequent retention and maturation to the T_{RM} phenotype (Mackay et al., 2013; Adachi et al., 2015). Analysis of TCR usage by

circulating and T_{RM} cells has revealed that T cells in the tissue appear to come from a subset of cells in circulation, though they are enriched for certain clones (Gaide et al., 2015). A majority of T_{RM} cell studies have focused on cells left at sites of inflammation once infection is resolved. While the pathways guiding migration of lymphocytes into sites of inflammation have been well established (Muller, 2013), the mechanisms by which cells enter uninflamed tissue sites have been poorly studied. Nonetheless, antigen-specific CD8+ T cells generated after viral infection have been shown to establish residency in unchallenged sites (Masopust et al., 2001; Jiang et al., 2012), and more recently there is evidence that CD4+ cells in the uninflamed skin can take on a tissue-resident phenotype (Bromley et al., 2012; Collins et al., 2016).

Here, we examine the requirements for entry of leishmania-specific CD4+ T cells into uninflamed skin sites after *L. major* challenge. We find that T_{RM} cells are present in uninflamed skin within 2 weeks of challenge, but are no longer generated once the infection is resolved. Upon rechallenge, leishmania memory cells regain the ability to form T_{RM} cells, suggesting that activated effector cells are crucial for this process. Indeed, when we compare effector and memory T cells directly, effector T cells are superior at skin entry, with this process appearing to be dependent on proliferation, and correlating with P and E selectin ligand expression. Fully defining the requirements for T cell entry into skin will allow the specific targeting of these cells in a vaccine.

4.3 Materials and Methods

Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee, University of Pennsylvania Animal Welfare Assurance Number 805186.

Mice

C57BL/6 mice were purchased from the National Cancer Institute (Fredericksburg, MD). Thy1.1/IFNγ reporter mice were obtained from Casey Weaver (University of Alabama Birmingham, Birmingham Alabama (Harrington et al., 2008)). All mice were maintained in a specific pathogen-free environment at the University of Pennsylvania Animal Care Facility.

Parasites

L. major (Friedlin) parasites were grown in complete Schneider's insect medium (GIBCO) supplemented with 20% heat-inactivated FBS, 2mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 50 µg/mL G418 sulfate (Cellgro) (CSM). Metacyclic enriched promastigotes were used for infection (Späth and Beverley, 2001). Mice were infected with 2 x 10^6 *L. major* or 10^3 *L. major* intradermally in the ear.

Antibodies

For flow cytometry analysis αCD45 APC-eF780, αCD45.2 FITC, αCD45.1PE-Cy7, αCD90.2 BV605, αCD11b BV650, αCD4 PE TexasRed, αCD8b PerCp/Cy5.5, αLy6C AF700, αLy6G PacBlue, αIFNγ PE-Cy7, P and E selectin APC, or αThy1.1 PE-Cy7 were incubated with single cell suspensions 30 minutes at 4°C and read on LSR Fortessa.

Skin Preparation

For ear preparation, dorsal and ventral cutaneous layers of the ear were separated and incubated in RPMI (Gibco) with 250 µg/mL Liberase TL (Roche) for 90 minutes at 37°C in 5% CO_2. Skin cells were then dissociated using a 40 µm cell strainer (BD Pharmingen) and resuspended in complete RPMI media (cRPMI) containing 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 55 µM 2-Mercaptoethanol. For flank skin preparation, a section of skin was harvested from the flank following hair removal with an electric trimmer equipped with a two-hole precision blade (Wahl). Skin sections were then minced with a sterile scalpel blade into ~2mm sections, and incubated in RPMI containing 1 mg each of type III and type IV collagenase (Worthington) for 120 minutes with vortexing every 30 minutes. The resulting solution was passed through a 40 µm cell strainer and resuspended in cRPMI. BMDCs for restimulations were generated by culturing C57BL/6 bone marrow in GM-CSF supplemented cRPMI for 7-11 days. BMDCs were then harvested and infected 5-8 hours with stationary phase *L. major* at a ratio of 10:1 in the presence of 1 µg/ml CpG and LPS. Infected BMDCs were incubated at a ratio of 1:5 with 10^6 skin cells in 24 well plates for 12-16 hours. Cells were incubated for the last 4 hours with 5 µg/ml BFA (eBioscience), stained for IFNγ, and analyzed by flow cytometry.

Skin grafts

Skin grafts were performed as previously described (Glennie et al., 2015). Briefly, donor skin was prepared under sterile conditions from naïve and immune mouse flank skin by shaving, depilating, cleaning with chlorhexidine (Vetoquinol), then excising the skin using sterile 8mm biopsy punches (Miltex). Grafts were placed onto a fresh graft bed prepared by excising skin using a 6mm biopsy punch. All mice were anesthetized, received

analgesics, and were monitored post-operatively as previously described. In challenge experiments, graft skin was injected intradermally with 2 x 10^6 metacyclic *L. major* 14-20 days after grafting.

Parabiosis

Congenically disparate mice were cohoused 2 weeks prior to surgery. After induction of anesthesia with isoflorane, each received 0.1mg/kg buprenorphine subcutaneously (s.q.) as preemptive analgesia. The surgical site was shaved and aseptically prepared with chlorhexidine scrub. A longitudinal skin incision was made on the mirroring side in each mouse starting at 0.5 cm above the elbow and ending 0.5 cm below the knee joint. The left elbow and knee of one animal were attached to the right elbow and knee of the other with a 3-0 ethilon suture (Ethicon) around each joint beneath the skin in a manner loose enough to not disrupt circulation to the distal limb. The dorsal and ventral skin edges created by the flank incision from one mouse were sutured to the respective skin edges of the second mouse using a continuous absorbable 5-0 vicryl suture patter (Ethicon). Suture glue (Abbott laboratories) was used to approximate skin edges. 0.5 ml of 0.9% NaCl was administered s.q. to each mouse to prevent dehydration in the immediate post-operative recovery period, and mice were monitored twice daily for the first 48 hrs post-operatively, then observed daily for signs of surgical site complications, pain, or discomfort. In challenge experiments, ears were infected intradermally with 2 x 10^6 metacyclic *L. major* 14-20 days after surgery.

Adoptive transfer

Single cell suspensions from spleen and lymph node were incubated 4 minutes at room temperature in the presence of 2.5 μM CFSE or CTV in the dark. The reaction was

quenched with 10% FBS and resuspended at 5-10 x 10^7 cells/mL in PBS, then transferred i.v. in 400 µL volume into recipient mice.

LCMV infection

C57BL/6 mice were injected i.p. with 2 x 10^5 pfu LCMV Armstrong. LCMV immune donor mice were sacrificed 5 or 40 days after infection, single cell suspensions were generated from spleen and lymph node, CFSE or CTV labeled, and transferred i.v. into recipient mice.

Statistical analysis

Statistical analysis was performed with the Mann-Whitney test (two-sided t-test, paired or unpaired where applicable), ANOVA, or 2-way ANOVA in Prism software (GraphPad).

4.4 Results

L. major-specific CD4+ T cells take up residency in uninflamed skin within 2 weeks of challenge

Mice that resolve a primary *L. major* infection develop an antigen-specific memory T cell response throughout the skin that appears to persist for the life of the animal (Glennie et al., 2015). To assess how early this population of T_{RM} cells forms after infection, we challenged WT C57BL/6 mice in the ear, waited 1 or 2 weeks after infection, then restimulated skin homogenates from the uninfected flank skin with *L. major* infected BMDCs (Fig. 1*A*). One week after infection, we were unable to detect a leishmania specific response above background (Fig. 1*B*), suggesting that T_{RM} cells had yet to form at this time. In contrast, there was a significant increase in leishmania-specific cells in

the flank skin 2 weeks post infection (Fig. 1*B*), indicating that leishmania-specific cells can enter uninflamed skin within the first 2 weeks of challenge.

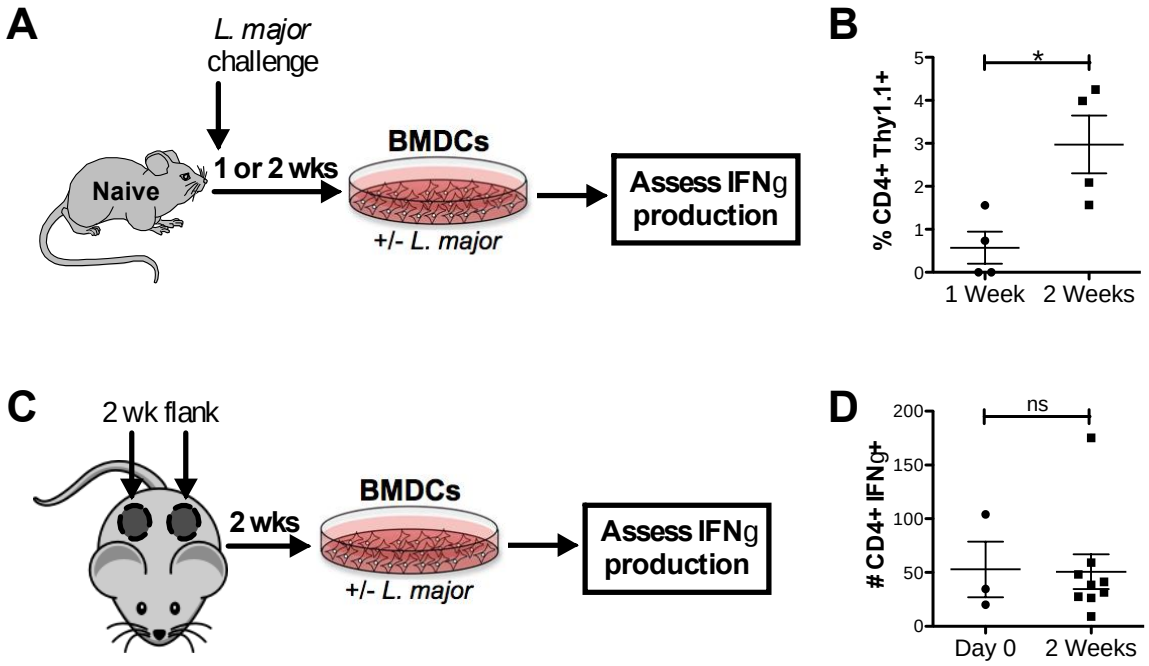

Figure 1. *L. major*-specific CD4+ T cells take up residency in uninflamed skin within 2 weeks of challenge. A) C57BL/6 mice were challenged intradermally in the ear with 2 x 10^6 *L. major.* 1 or 2 weeks after challenge, flank skin was harvested, restimulated with *L. major* infected BMDCs, and leishmania-specific cells were assessed by IFNγ production. B) The frequency of IFNγ producing CD4+ T cells in flank skin 1 or 2 weeks after challenge as represented by Thy1.1 surface expression in IFNγ/Thy1.1 knock-in reporter mice (Harrington et al. 2008). C) Skin from mice that had been infected for 2 weeks was grafted onto naïve recipients. 2 weeks later, cells from the graft skin were restimulated with *L. major* antigen, and antigen specific responses were measured by IFNγ production. D) The number of leishmania-specific T cells contained in skin grafts at the time of grafting (day 0), and 2 weeks after skin grafting.

To test if these leishmania-specific cells in flank skin 2 weeks after infection were forming a tissue-resident population, we performed skin grafts in which skin from the flanks of mice that had been infected 2 weeks earlier were grafted onto naïve recipients. After allowing 2 weeks for the grafts to heal and immune cells to equilibrate, we

harvested the grafts, restimulated with *L. major* antigens, and assessed leishmania-specific CD4+ T cell responses by IFNγ production (Fig. 1C). Strikingly, we observed the same number of cells in the skin grafts 2 weeks after transplant, suggesting that these cells did not recirculate. Together, these data demonstrate that leishmania-specific CD4+ T cells enter uninflamed skin sites within 2 weeks of challenge, and even at this early time point they are capable of establishing tissue-residency.

L. major-specific T cells no longer enter uninflamed skin once the infection is resolved

Having established that T_{RM} cells can enter uninflamed skin very early after infection, we next wanted to assess their ability to continually enter uninflamed skin once the infection is resolved. To do so, we used parabiosis in which we surgically linked the circulations of naïve and immune mice that had resolved a primary infection. As expected, we observed equal sharing of lymphocyte populations in the blood 2 weeks after joining, as represented by the frequencies of naïve (CD45.2) and immune (CD45.1) origin cells recovered from blood of the naïve animals (Fig. 2*A*). Importantly, when we restimulated cells from the blood with *L. major* infected BMDCs, we also saw equal mixing of IFNγ+, leishmania-specific CD4+ T cells (Fig. 2B). Strikingly, although we saw some entry of total CD4+ cells into the flank skin, IFNγ+ leishmania-specific cells could only be recovered from the flanks of the immune mice, suggesting that memory cells are unable to efficiently enter naïve skin, at least within the 2 weeks of shared circulation.

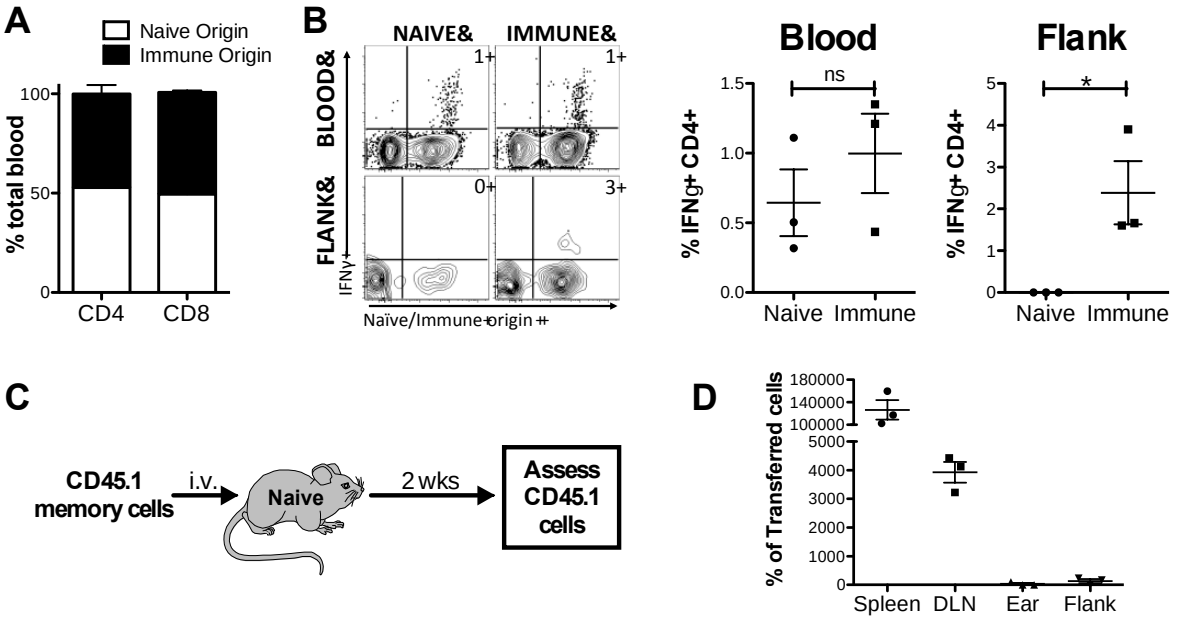

Figure 2. *L. major*-specific effector T cells no longer enter non-inflamed skin once infection is resolved. A) Proportions of CD4+ and CD8+ T cells of naïve (white) or immune (black) origin found in naïve parabionts 2.5 weeks after joining. B) Representative and combined data showing frequency of leishmania-specific, IFNγ+ cells in the blood and flank of naïve and immune parabionts 2.5 weeks after surgery upon restimulation with *L. major* infected BMDCs. C) Total splenocytes from CD45.1 immune mice were transferred into naïve CD45.2 recipient mice. 2 weeks later, the distribution of transferred CD4+ cells in spleen, DLN, ear, and flank were assessed by flow cytometry. D) The number of transferred cells recovered from spleen, DLN, ear, and flank 2 weeks after transfer.

To confirm that memory T cells were unable to efficiently enter uninflamed skin sites, we directly transferred labeled (CD45.1) splenocytes from immune mice, which are enriched for leishmania-specific CD4+ T cells, into naïve (CD45.2) recipient mice. 2 weeks later, we harvested various tissue sites to assess the migration of the transferred CD4+ cells (Fig 2*C*). As expected, we found significant populations of transferred CD4+ cells in the spleen and cervical draining lymph nodes of all recipient mice (Fig. 2*D*). In contrast, we saw virtually no infiltration of the transferred cells into either the ear or the flank skin

within 2 weeks (Fig. 2*D*), again suggesting that CD4+ cells from the memory phase of infection are inefficient at trafficking into uninflamed skin.

Memory cells regain the ability to enter uninflamed skin following rechallenge

We next wanted to ask if leishmania memory cells would regain the ability to enter uninflamed skin upon restimulation with antigen. To do so, we set up naïve and immune parabiotic pairs to ensure we would have a physiologic level of circulating memory cells with access to naïve skin. We then challenged each mouse intradermally in the ear with *L. major*, and 72 hours later assessed the migration of leishmania-specific CD4+ T cells into the flank skin compared with unchallenged parabionts (Fig. 3*A*). As previously observed, in the absence of challenge we did not see any migration of cells into naïve flank skin, while we did observe a population of T_{RM} cells in the immune mice (Fig. 3*B*). Strikingly, upon challenge we not only observed a population of cells that could infiltrate the naïve skin, but also observed an increase in the number of responsive cells in the immune flanks (Fig. 3*B*). These data suggest that rechallenge does indeed generate a population of effector cells that infiltrate naïve skin and boost responses in immune skin. Moreover, this change happens very rapidly, and can be observed within 72 hours of restimulation.

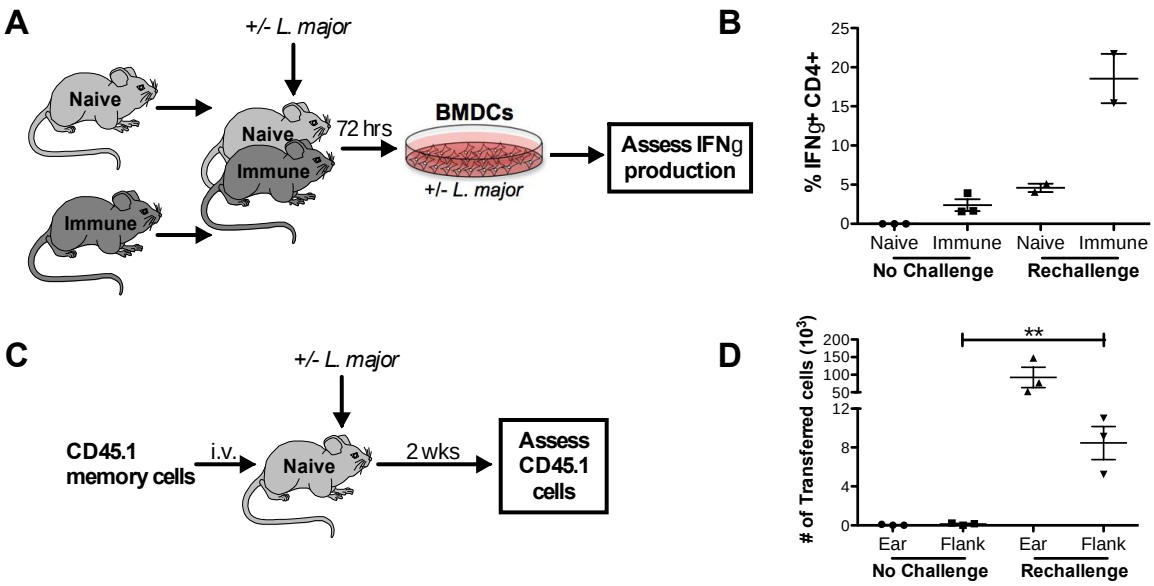

Figure 3. Memory cells regain the ability to enter uninflamed skin following rechallenge. A) Naïve and immune mice were linked by parabiotic surgery. 2 weeks later, each naïve and immune mouse was challenged in the contralateral ear, and then leishmania-specific responses were assessed by *in vitro* restimulation with *L. major* infected BMDCs. B) Frequency of leishmania-specific cells in the flank skin of naïve and immune parabionts with or without rechallenge 72 hrs earlier. C) Total splenocytes from CD45.1 immune mice were transferred into naïve CD45.2 recipient mice with or without *L. major* challenge. 2 weeks later, the distribution of transferred CD4+ cells in the ear and flank skin were assessed by flow cytometry. D) The number of transferred cells recovered from ear and flank skin with and without *L. major* challenge 2 weeks after transfer.

To test this hypothesis another way, we adoptively transferred labeled (CD45.1) total splenocytes from immune mice into naïve (CD45.2) recipients, challenged intradermally in the ear with *L. major*, and 2 weeks later measured the infiltration of transferred cells into the skin (Fig. 3*C*). As previously observed, in the absence of *L. major* challenge leishmania memory cells did not significantly infiltrate ear or flank skin (Fig. 3*D*). As expected, 2 weeks after challenge we were able to recover many cells from the challenged ear, suggesting that these cells were capable of responding to inflammation (Fig. 3*D*). Again, we also found a significant infiltration of our transferred cells into

uninflamed flank skin, indicating that restimulation of leishmania memory cells is sufficient for allowing them to regain the ability to enter uninflamed skin sites.

Recently activated effector cells are superior to memory cells at entering uninflamed skin

These findings suggest that recently activated effector cells are superior to memory cells at forming T_{RM} cells because they are better able to enter uninflamed skin sites. To confirm that the cells entering uninflamed sites were indeed recently activated, we used CFSE labeling dye to assess T cell proliferation in response to antigen stimulation. As before, we transferred congenically disparate, CFSE labeled cells into naïve mice, challenged with *L. major*, and 2 weeks later examined CFSE dilution on cells in various tissues (Fig. 4*A*). As expected, there was very low background proliferation of transferred cells in the skin draining lymph node of mice that were not challenged (Fig. 4*B*). In contrast, cells from the draining lymph node of challenged mice showed robust, presumably antigen-specific proliferation, causing roughly half the cells to dilute their CFSE (Fig. 4*B*). Convincingly, virtually all the cells we were able to detect in the uninflamed flank skin had completely diluted CFSE (Fig. 4*B*), indicating that they indeed had been recently activated, and suggesting that this may be a requirement for entry into uninflamed skin.

P and E selectin ligands (PESL) are expressed by activated T cells, and augment entry into inflamed skin sites by binding to their receptors on the surface of activated endothelial cells, subsequently leading to extravasation into the tissue (Tietz et al., 1998). We therefore examined the expression of P and E selectin ligands on transferred CD4+ cells that we recovered from various sites to determine if they were associated

with activation or tissue entry. PESL expression was very low on CD4+ cells in secondary lymphoid organs, consistent with a less activated phenotype (Fig. 4*C*). By comparison, CD4+ cells that infiltrated the challenged ear had relatively higher expression of PESL, though they were still only expressed on roughly 30% of cells (Fig. 4*C*). In contrast, greater than 90% of CD4+ cells that entered the uninflamed flank skin stained positive for PESL (Fig. 4*C*), indicating that these cells have not only proliferated, but they have also upregulated molecules associated with entry into skin.

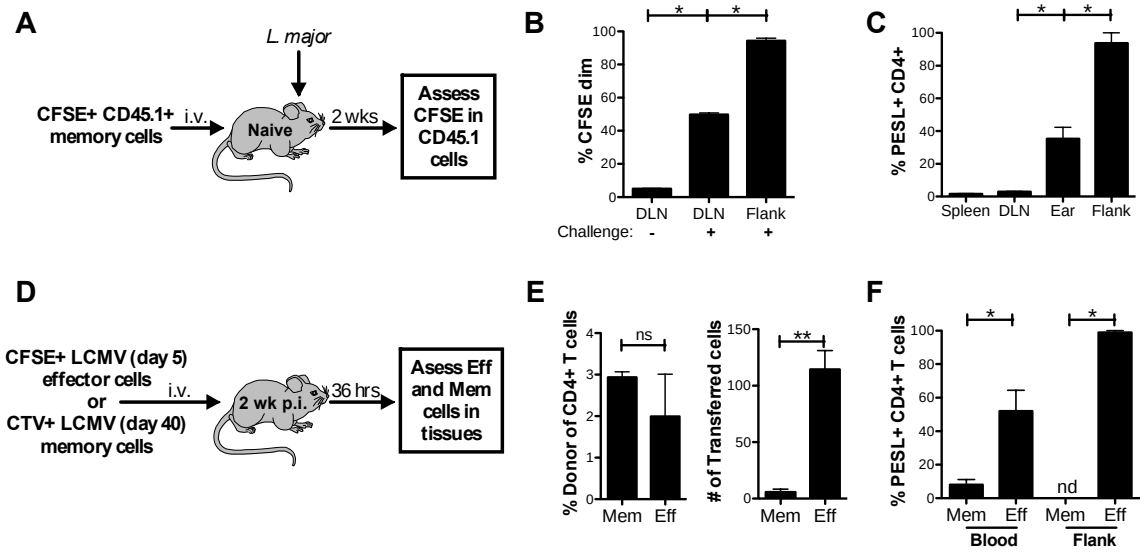

Figure 4. Recently activated effector cells are superior to memory cells at entering uninflamed skin. A) CFSE labeled splenocytes from leishmania immune mice were adoptively transferred into naïve recipients that were then challenged with *L. major*. 2 weeks later, CFSE and PESL expression on transferred cells were measured in various tissue sites. B) The frequency of transferred cells that had diluted CFSE in challenged and unchallenged DLNs, as well as the flank skin of mice 2 weeks post challenge. C) The frequency of transferred CD4+ cells expressing PESL in spleen, DLN, ear, and flank 2 weeks after *L. major* challenge. D) Total splenocytes from LCMV infected mice at the effector phase (day 5) or memory phase (day 40) were labeled with CFSE or CTV respectively, then adoptively transferred into 2 week post *L. major* infected recipients. 36 hours later, the migration and PESL expression on transferred cells was examined in the blood and flank skin. E) The frequency of LCMV memory or effector phase cells recovered from the blood (left) or flank (right) 36 hours after transfer. F) The frequency of LCMV memory and effector phase cells expressing PESL in the blood or flank skin 36 hours after transfer.

To test if this phenomenon was specific to *L. major* infection, and take advantage of a system in which the kinetics of infection are better defined, we next directly compared the migration of LCMV specific effector and memory phase CD4+ T cells. To do so, we isolated total splenocytes from LCMV infected mice at peak infection (5 dpi) when they would have a large population of effector phenotype cells, and in the memory phase (40 dpi) when the virus is totally cleared and responding T cells have taken on a memory phenotype (Fig. 4*D*). We then transferred these cells in equal numbers into mice that were 2 weeks post *L. major* infection, and assessed their migration into tissue 36 hrs later by flow cytometry (Fig. 4*D*). Importantly, we observed similar frequencies of our transferred CD4+ cells in the blood (Fig. 4*E*, left), indicating that we transferred similar numbers of cells. However, when we examined the number of cells of each phenotype in the flank skin, we saw that only effector cells were capable of migrating in, while virtually no memory cells could be detected (Fig. 4*E*, right). Additionally, when we examined PESL expression, effector phase CD4+ cells had significantly higher expression both in the blood and even more so in the flank skin (Fig. 4*F*). Taken together, these data further indicate that recently activated effector cells are capable of infiltrating uninflamed skin sites, while quiescent memory cells are not, and that the ability to do so correlates with activation markers, such as PESL expression.

4.5 Discussion

Despite a number of examples of T_{RM} cells providing rapid protection against secondary infection, and a number of descriptions of the requirements for retention of CD8+ T cells (Mackay et al., 2013) and even CD4+ T cells (Adachi et al., 2015; Iijima and Iwasaki, 2014) in tissue, relatively little is known about how T_{RM} cells enter tissue, particularly at

sites distal to infection. Here, we study leishmania-specific T_{RM} cells to try and elucidate some of the mechanisms by which these cells gain entry into the skin, specifically in uninflamed sites. Strikingly, we find that entry occurs as early as 2 weeks after infection, but seems to be dramatically restricted once infection is resolved. Further, we find that proliferated effector cells are far superior to memory cells at entering uninflamed skin, suggesting that these are the cells to target for inducing T_{RM} cells.

It has been appreciated that CD4+ T cells are much more mobile in the skin than CD8+ T cells, correlating with their predilection for localizing to the dermis, while CD8+ T cells are more likely to be in the epidermis (Gebhardt et al., 2011). Moreover, CD4+ T cells are much more likely to be found migrating out of the skin at steady state (Debes et al., 2006; Bromley et al., 2012). Together, these data suggest that CD4+ T cells are much more migratory within tertiary tissues, and thus may be more inclined to enter uninflamed skin than other lymphocytes. Indeed, it has been observed that if you wait long enough after parabiosis, CD4+ cells in the skin will completely equilibrate in naïve SPF mice, suggesting that they have a significant ability to migrate through tissue (Collins et al., 2016). Nonetheless, a number of different CD4+ T_{RM} cell populations have been identified (Iijima and Iwasaki, 2014; Laidlaw et al., 2014; Collins et al., 2016; Teijaro et al., 2011), and we have identified a population of non-circulating leishmania-specific T_{RM} cells in the skin (Glennie et al., 2015, 2017). Together, these data suggest that while CD4+ cells have an ability to migrate relatively well in tissue, particularly in comparison to their CD8+ counterparts, infection induced changes in the T cells, the environment, or some combination of the two licenses these CD4+ cells to enter tissue and establish residency. Our findings that highly proliferated, activated effector cells are the cells most likely to enter uninflamed tissue indicate that there may be T cell intrinsic

changes that increase their ability to enter, which presumably leads to their increased retention in the skin. Going forward, we will need to validate that those cells that enter skin are in fact retained, though based on our skin graft results they appear to be retained for at least 2 weeks after grafting.

Unlike other tissues, the skin is constantly in direct contact with the exterior environment. Factors such as the skin microbiome and mechanical disruption caused by physical contact have the potential to influence T_{RM} cell generation. In fact, colonization of mouse skin with a single bacteria species has been shown to increase the generation of some T_{RM} cell populations in specific contexts (Naik et al., 2015, 2012). In vaccinia infection, skin-scarification appears to increase the generation of T_{RM} cells (Liu et al., 2010; Jiang et al., 2012), but the extent to which physical injury might promote the generation of T_{RM} cells at sites distal to the site of infection remains to be fully examined. Interestingly, the topography of hair follicles and signals derived from hair follicle-associated microbiota may be important for T_{RM} cell maintenance (Adachi et al., 2015), but the contributions of these factors in human skin remain to be defined.

Intriguingly, we found that rechallenge of parabiotic pairs containing one naïve and one immune animal not only led to the generation of a leishmania-specific skin response in the naïve animals, but also appeared to boost the existing T_{RM} response in the immune parabionts. This is exciting, because it suggests that boosting via vaccination, or even through naturally occurring reinfection, may lead to an increase in T_{RM} cells and thus an increase in protective immunity. In future studies, it will be useful to determine the extent to which repeated inoculation increases skin-resident as well as circulating immune responses in order to optimize a potential vaccine strategy. From this study, one can

conclude that multiple rounds of boosting and the generation of large pools of highly proliferated, recently activated effector cells may be critical for a vaccine to generate T_{RM} cells. Further defining the exact transcription factors and surface molecules required for skin entry will be required to fully understand how these cells are formed, and to allow them to be leveraged in a vaccine.

CHAPTER 5: DISCUSSION

5.1 Developing a cutaneous leishmaniasis vaccine

Cutaneous leishmaniasis poses a conundrum for vaccine development. On the one hand, control of a primary infection is highly protective, a prospect that bodes well for any potential vaccine. On the other hand, the parasite is never fully cleared, and forcing elimination of the parasite reduces immunity to reinfection (Uzonna et al., 2001; Belkaid et al., 2002a). While a live vaccine might provide the best protection, it may be difficult to deliver such a vaccine due to ethical and logistical concerns. Encouragingly, it is not required that a cutaneous leishmaniasis vaccine provides sterile immunity, as it is apparent that low levels of parasites can be maintained without pathology. Therefore, the goal of a cutaneous leishmaniasis vaccine might not be to completely eliminate the parasites but instead reduce the parasite load below a certain threshold, and in particular decrease the incidence of the most pathologic outcomes of disease such as mucosal, disseminated, and diffuse cutaneous leishmaniasis.

Circulating CD4+ T_{Eff} cells are potent mediators of immunity and thus are attractive to target in a vaccine. Unfortunately, these T cells appear to require parasites to persist,

and therefore maintaining them will rely on a strategy that supplies continuous antigen either through a live-attenuated pathogen, frequent boosts, or some other persistent delivery platform. The development of a safe live-attenuated parasite that maintains T_{Eff}, T_{CM}, and T_{RM} may be the simplest path to an effective vaccine, but has proven difficult to develop (Kedzierski et al., 2006; Palatnik-de-Sousa, 2008). Another barrier to the generation of a CD4+ T cell vaccine has been the lack of well-defined immunodominant CD4+ T cell epitopes, as most immunization strategies have required complex polyprotein constructs to induce responses (Costa et al., 2011; Duthie et al., 2016). The recent identification of a protein, phosphoenolpyruvate carboxykinase, that reacts with approximately 20% of *L. major*-specific CD4+ T cells and has broad cross-reactivity may help resolve this need, and will also be an invaluable tool for assessing CD4+ responses (Mou et al., 2015). Nevertheless, continuing efforts to discover conserved, immunostimulatory antigens may be necessary to get the coverage required for robust vaccine-induced immunity.

Fortunately, while T_{Eff} cells require persistent antigen, there are at least two populations of memory T cells that are maintained in the absence of persistent parasites. T_{CM} cells persist and alone can provide some resistance in mice, though the protection is delayed compared to T_{Eff} cells (Zaph et al., 2004). CCR7+ CD4+ memory cells that show increased proliferative capacity in response to leishmania antigen have also been identified in cutaneous leishmaniasis patients that have healed (Keshavarz Valian et al., 2013), suggesting that T_{CM} cells are generated in humans as well.

T_{RM} cells have never been targeted in a leishmania vaccine, and may be the missing link for optimal vaccine-induced protection. Indeed, when challenged by sand fly bite instead

of needle injection, only immune mice and not vaccinated mice are protected against *L. major* (Peters et al., 2009). Since immune mice contain a population of T_{RM} cells, this result may demonstrate the importance of T_{RM} cells for a vaccine. Our finding that T_{RM} cells mediate the DTH response in mice (Glennie et al., 2017), and the fact that the DTH response correlates with protection in mice and in humans, additionally suggests that T_{RM} cells may have a critical role to play (Rossell et al., 1987; Sassi et al., 1999; Schnorr et al., 2012). Intriguingly, since all species of the parasite are transmitted through the skin, T_{RM} cells may be able to provide significant cross-protection, even against the visceral form of the disease. The hallmark of T_{RM} cell-mediated protection is a rapid response, which may be critical for controlling the parasites before they have time to replicate and expand extensively. Since T_{RM} cells respond rapidly and T_{CM} cells maintain a reservoir of responsive cells, a vaccine that combines the induction of T_{RM} cells and circulating T_{CM} responses may offer the most realistic hope of a safe, effective vaccine for cutaneous leishmaniasis.

5.2 Mechanisms of T_{RM} induced protection

There are several potential mechanisms by which Trm cells could provide protection against a challenge infection. While one function of CD8+ T_{RM} cells appears to be production of effector molecules that can directly kill target cells (Kim et al., 1999; Masopust et al., 2006; Steinbach et al., 2016), most of the identified T_{RM} cell functions are mediated indirectly through downstream effects of inflammatory cytokine production. For example, recent studies of CD8+ T_{RM} cells in the female reproductive tract demonstrated IFNγ-dependent B and T cell recruitment (Schenkel et al., 2013), along with local DC maturation, and NK cell activation (Schenkel, 2014). Several studies have

demonstrated broad transcriptional changes occurring in tissue after T_{RM} cell activation that result in protection (Schenkel, 2014; Ariotti et al., 2014; Glennie et al., 2015), but relatively few have delved into the details of how this protection is mediated.

We have identified two specific effector functions of T_{RM} cells during cutaneous leishmaniasis: 1) To promote CXCR3-dependent recruitment of circulating effector T cells, and 2) To activate an enhanced innate inflammatory response and CCR2-dependent recruitment of inflammatory monocytes. Our findings extend those from CD8+ T_{RM} cells in the FRT to CD4+ T_{RM} cells in the skin, and this process probably occurs by a similar mechanism, which may be at least in part mediated by activation of the endothelium and upregulation of VCAM-1 (Schenkel, 2014). This result is interesting because it demonstrates for the first time that a CD4+ T_{RM} population can promote T cell recruitment, and it suggests that even a small number of cells distributed broadly throughout the tissue can mobilize a much larger adaptive immune response by calling circulating T cells into the tissue to perpetuate the response.

More interestingly, we have also identified a novel mechanism by which T_{RM} cells function in recruiting inflammatory monocytes into the tissue, resulting in direct control of a pathogen (Glennie et al., 2017). While other groups have suggested or demonstrated interactions between T_{RM} cells and innate cells (Iijima and Iwasaki, 2014; Schenkel, 2014; Adachi et al., 2015; Collins et al., 2016), none to our knowledge have shown T_{RM} cell-mediated protection through innate cell recruitment and activation. This discovery may have a broad impact on a number of different infections in which inflammatory monocytes promote protection (Lim et al., 2011; Aldridge et al., 2009; Serbina et al., 2003; Peters et al., 2001; Moyat et al., 2015; Hohl et al., 2009; John J. Osterholzer,

Gwo-Hsiao Chen, Michal A. Olsewski, Jeffrey L. Curtis, Gary B. Huffnagle, 2014; Robben et al., 2005; Strauss-Ayali et al., 2007; León et al., 2007). Further, our finding that T_{RM} cell driven recruitment of inflammatory monocytes is responsible for the DTH response extends findings from contact hypersensitivity models (Adachi et al., 2015; Gaide et al., 2015), and suggests that this correlate of adaptive immunity is also dependent on T_{RM} cells and not circulating cells, which has implications for a number of diseases. Several questions remain going forward, including what T cell function initiates the signaling cascade that leads to inflammatory monocyte recruitment, the cell types involved in inflammatory chemokine production, and the relative roles of ROS and NO in protection. Further elucidation of additional cytokine dependent T_{RM} cell functions, as well as functions that might be mediated by direct cell-to-cell contact is warranted.

5.3 Inducing and maintaining T_{RM} cells

If T_{RM} cells in fact offer hope as a vaccine strategy, then it is critical that we understand the requirements for the induction and maintenance of T_{RM} cell populations. Unfortunately, it is still poorly understood how to target T_{RM} cells through vaccination, although one important factor may be the route of inoculation. Skin-scarification has been shown to induce increased proliferation of T cells in skin-draining lymph nodes, as well as increased homing to peripheral tissue (Liu et al., 2010; Hersperger et al., 2014). These studies suggest that a delivery strategy involving epidermal abrasion may generate T_{RM} cells more effectively than other delivery methods, though the exact mechanisms by which this occurs still remain unclear.

We have explored this concept in a preliminary study using two different methods of DNA vaccination. Intramuscular DNA vaccination with subsequent electroporation has been shown to generate increased uptake of DNA and expression of protein antigens, subsequently leading to stronger CD4+, CD8+, and antibody responses against the target antigen (Sardesai and Weiner, 2011). Intradermal DNA electroporation involves the injection of DNA directly into the skin, followed by the delivery of an electronic pulse which is designed to increase uptake of the DNA, but may also cause some level of tissue damage. Our preliminary data suggest that antigen delivered via intradermal vaccination generates significantly better cutaneous CD4+ responses both at the site of vaccination, and at distant skin sites (Fig. 1). This result reaffirms that vaccination via the tissue of interest may be important for T_{RM} cell generation. As a natural infection model that generates skin-associated T_{RM} cell responses not just at the infection site but also in uninflamed skin sites, leishmaniasis offers an opportunity to further understand the requirements for generating T_{RM} cells.

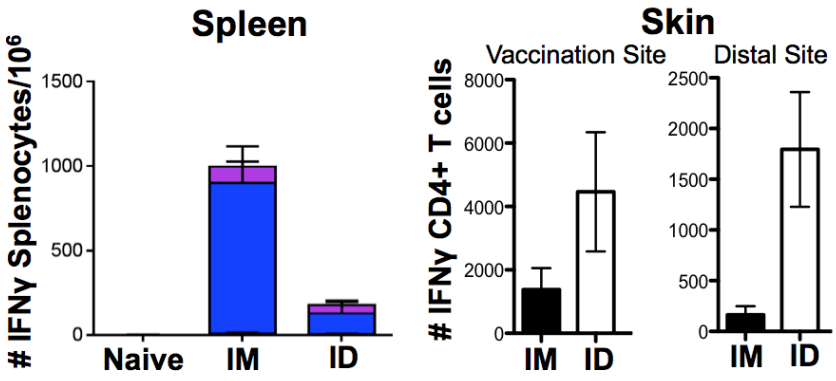

Figure 1. Intradermal DNA electroporation is superior to intramuscular at eliciting skin-associated T cell responses. (Left) The number of vaccine-specific IFNγ-producing splenocytes was determined by Elispot 24 days after vaccination of C57BL/6 mice either intramuscularly (IM) or intradermally (ID). (Right) In the same mice, the number of vaccine-specific IFNγ-producing T cells

in the skin at the site of vaccination, or at a distal, contralateral site were quantified by flow cytometry 24 days after vaccination.

Continuing to explore mechanisms that promote the infiltration and maintenance of antigen-specific cells into the tissue will be critical for promoting T_{RM} cell populations. Another method that has seen some success is the topical application of chemokines. Specifically, the application of CXCL9 and CXCL10, which drive recruitment and retention of CXCR3+ effector T cells, directly into the female reproductive tract promoted the migration of HSV-2 specific T cells into the tissue, which subsequently provided enhanced protection against challenge (Shin and Iwasaki, 2012). This "prime and pull" method of vaccination, though perhaps impractical in humans, represents a proof of principle that the artificial augmentation of inflammatory cues in the tissue may drive increased T_{RM} cell formation.

One critical question to understand is what type of T cells give rise to T_{RM} cells, specifically whether effector or memory phenotype T cells are better at forming T_{RM} cell populations. In chapter 4 we investigated this question in the leishmania infection model, and discovered that recently activated, recently proliferated, and PESL+ cells appeared to best be able to enter skin, and remain resident there for at least 2 weeks. However, it has been proposed in other models that pre-T_{RM} cells are in fact less highly differentiated cells, allowing them to take on a memory phenotype once they lodge in tissue (Sheridan et al., 2014). On the other hand, emerging evidence indicates that T_{RM} cells more closely mimic T effector cells, which may suggest that they form from effector T cell precursors (Mackay et al., 2016, 2013; Gaide et al., 2015). Continuing to dissect what type of T cells best form T_{RM} cells will be important for generating them experimentally.

Moreover, the extent to which T cell intrinsic factors versus environmental factors drive T_{RM} cell formation remains to be defined. Some studies, including ours, have suggested that there may be inherent differences between the T cells that do or do not enter tissue (Gaide et al., 2015). However, there is ample evidence that once in the tissue, specific tissue-derived signals augment or maintain T_{RM} cell populations, and lead to specific changes in T_{RM} cell phenotype (Masopust et al., 2006; Mackay et al., 2013; Iijima and Iwasaki, 2014; Adachi et al., 2015). Additionally, there may be infection-induced changes in the epithelium, resulting in enhanced T cell infiltration regardless of T cell phenotype (Iijima and Iwasaki, 2015; Ebel et al., 2015; Schenkel, 2014). Defining the extent to which each process plays a role in T_{RM} cell generation and maintenance is important to our understanding of these cells.

Similarly, the role that antigen might play in the establishment and maintenance of T_{RM} cells in the tissue remains ill-defined. In leishmaniasis, where primary infection results in the chronic persistence of parasites at the site of infection and in the draining lymph node, antigen will always be present in an immune animal, though it does not persist in sites where resident memory cells form (Nicolas et al., 2000). Based on our skin graft results, antigen does not appear to be required for parasites to persist at least 4 weeks after transfer (Glennie et al., 2015), though this does not rule out a role for antigen in the formation of T_{RM} cell populations. Indeed, a recent study nicely demonstrated that the recognition of cognate antigen within tissue can substantially augment the formation and establishment of residency within tissue (Khan et al., 2016). However, several other studies have demonstrated that T_{RM} cell formation does not rely on the presence of antigen, and that T_{RM} cells can be maintained in the absence of any persistent antigen (Casey et al., 2012; Mackay et al., 2012). Undoubtedly, the requirement for antigen may

vary based on the infection, infection site, and the tissues involved. Nevertheless, there is certainly a role that antigen can play both in the initiation of T_{RM} cell forming responses, and in augmenting T_{RM} cell responses in the tissue. However, since antigen is not absolutely required for the maintenance of T_{RM} cell populations, there is hope that these cells can be retained even when antigen levels fade, as occurs during vaccination.

Many studies have sought to establish specific factors associated with T_{RM} cells, with varying degrees of success. Initially, it appeared that integrin-α component CD103 would be a definitive marker for tissue-resident cells (Kim et al., 1999; Schön et al., 1999). Subsequent studies, however, revealed that not only was it less ubiquitously expressed by CD4+ compared to CD8+ T_{RM} cells (Teijaro et al., 2011; Shin and Iwasaki, 2012; Laidlaw et al., 2014), but also that CD103- CD8+ T_{RM} cells could exist as well (Anderson et al., 2012; Bergsbaken and Bevan, 2015). Upregulation of the CD69 axis and associated reduction in surface receptor S1P1R, as well as transcription factor KLF2 have also appeared to be good candidates as T_{RM} cell markers, and in many cases do associate with T_{RM} cell formation (Masopust et al., 2006; Skon et al., 2013), but follow-up studies have demonstrated that these too are imperfect markers (Anderson et al., 2014; Watanabe et al., 2015). Genome wide expression profiling has revealed that there are some signatures that associate specifically with T_{RM} cells across a variety of infections and a variety of tissues (Mackay et al., 2013). Recently, transcription factors Blimp-1 and Hobit have been shown to specifically promote the retention of CD8+ T_{RM} cells in tissue (Mackay et al., 2016). Preliminary data from leishmania infected mice suggest that these factors may also associate with CD4+ T_{RM} cells, though follow up studies will be needed to confirm these data (Fig. 2). Although it is inevitable that variability among infections, tissue site, and cell type will influence which specific markers are associated

with T_{RM} cells, as we continue to gain more insight into T_{RM} cell phenotypes it is possible that a consensus core signature will emerge

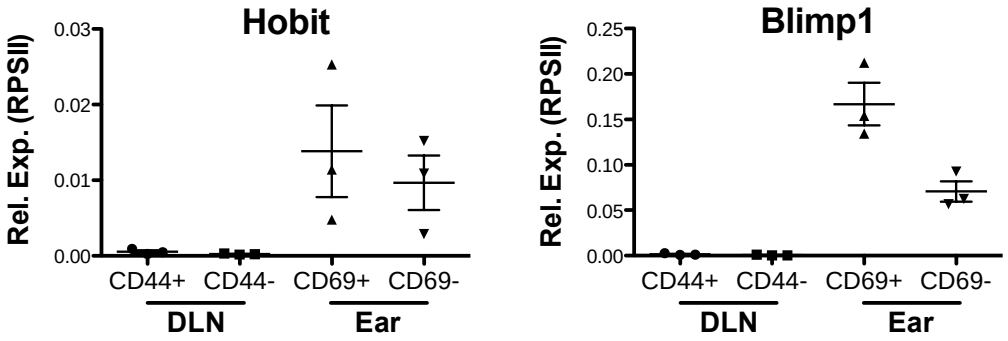

Figure 2. Hobit and Blimp-1 are transcription factors associated with CD69+ CD4+ T cells in the skin. CD4+ T cells from the draining lymph node or primary ear of 7 week infected mice were further sorted by activation status using CD44 in the DLN or CD69 in the skin. Hobit and Blimp-1 expression levels were examined by qPCR.

5.4 T_{RM} cells in disease

T_{RM} cells have proven to be critical mediators of protection in a number of different contexts. However, as mediators of tissue inflammation, they also have the potential to drive pathologic effects. For example, in cutaneous T cell lymphoma, resistance against treatment with the T cell depleting antibody Alemtuzumab (αCD52) correlates with the persistence of a population of T_{RM} cells that can reactivate disease once treatment is stopped (Clark et al., 2012; Watanabe et al., 2014). Similarly, human psoriatic lesions often recur in the same places even after treatment, and areas of recurrent disease are also associated with a population of highly activated, T_{RM} phenotype cells that rapidly produce IL-17A and IL-22 in response to *ex vivo* restimulation (Cheuk et al., 2014). Finally, it has recently been identified that a population of allergen-specific, lung-resident CD4+ T cells is sufficient to drive asthmatic disease in response to antigen re-exposure

(Hondowicz et al., 2015). Together, these data indicate that while T_{RM} cells can provide rapid and robust protection in the right contexts, in situations of aberrant or exacerbated inflammation they also have the potential to directly mediate pathology.

The pathologic potential of T_{RM} cells has important implications for leishmaniasis, a complex and spectral disease in which immunopathology is often an issue. Our lab alone has identified multiple contexts in which CD8+ T cells can be pathologic: 1) In driving metastatic disease and pathology after *L. braziliensis* infection (Novais et al., 2013) and, 2) As activated bystander cells that contribute to exacerbated inflammation in an NKG2D-dependent manner (Crosby et al., 2014, 2015). If either of these CD8+ T cell subsets were to form a tissue resident population, they would be poised to contribute to secondary disease. With such a diverse array of clinical responses to infection, it is very possible that leishmania-specific or bystander T_{RM} cells could be contributing to at least a subset of these adverse inflammatory outcomes, though the contributions of T_{RM} cells to human leishmaniasis have not been studied. Nonetheless, CD8+ T cell-mediated inflammatory processes are highly upregulated in leishmania lesions (Novais et al., 2013). We have not identified any IFNγ-producing CD8+ T_{RM} cells in the mouse model (data not shown), but whether or not a population of CD8+ T_{RM} cells exists that may perform another protective effector function remains to be determined. CD8+ T cells have also previously been shown to be important for secondary protection in several contexts (Belkaid et al., 2002b; Müller et al., 1994), and thus whether or not leishmania-specific CD8+ T cells form T_{RM} cell populations, and the role they might play during secondary infection, should be further studied.

5.5 Unresolved questions about T_{RM} cells

It is now clear that T lymphocytes in non-lymphoid tissue have an important role to play in infection induced immunity, autoimmunity, and potentially vaccination. However, there are still a number of unresolved questions surrounding these cells, suggesting that the way we think about them may change significantly as we continue to gain insight into mechanisms of their development, maintenance, and method of action. Issues surrounding the development of T_{RM} cells including the role of antigen, T cell intrinsic qualities, T_{RM} cell functions, and tissue-specific signaling have been previously discussed in this text (Section 5.2). Similarly, a number of questions remain poorly addressed regarding the long-term maintenance of T_{RM} cells in tissue.

Few studies have rigorously addressed the question of how long T_{RM} cells are maintained in tissue after infection. At a population level, T_{RM} cell populations appear to be maintained at relatively stable frequencies throughout the life of a mouse (Masopust et al., 2001; Jiang et al., 2012). Similarly in our studies, we see leishmania-specific T_{RM} cells maintained for roughly a year after the resolution of disease (Glennie et al., 2015), but whether or not these cells are being reseeded from circulation remains to be determined by long term skin graft or parabiosis studies. In parabiosis studies that have long-term (24 weeks) post-operative follow-up, T_{RM} cells have appeared to remain relatively stable and not repopulate the naïve parabiont at any appreciable frequency (Jiang et al., 2012). However, more studies of this nature will need to confirm these findings, as there are likely tissue and infection specific differences.

If T_{RM} cells are not being reseeded from circulation, then they are either extremely long-lived and/or undergoing homeostatic turnover. Several T cell survival factors such as IL-

7, IL-15, the aryl hydrocarbon receptor, and BCL-2 have been associated with T_{RM} cells (Adachi et al., 2015; Schenkel et al., 2014b; Mackay et al., 2013; Zaid et al., 2014), suggesting that they may indeed be able to survive for long periods in the tissue. However, there appear to be tissue specific differences as CD8+ T_{RM} cells in the female reproductive tract could be retained independently of IL-15, while cells in the skin could not (Schenkel et al., 2016; Adachi et al., 2015). One study has demonstrated that T_{RM} cells in the brain undergo homeostatic proliferation, as indicated by Ki-67 staining and STAT5 phosphorylation. Further study will be required to determine if this is a consistent feature of T_{RM} cells.

Another understudied area is the impact of the microbiome on T_{RM} cell populations. It is clear in the gut of both mice and humans that the composition of the microbiome effects mucosal and systemic immune responses (Smolinska et al., 2017; Schirmer et al., 2016). It seems likely that the microbiome will potentially play a role in the establishment, maintenance, or activation of T_{RM} cells, but little work has yet been done in these areas. A series of studies in the gut links the generation of different T_{RM} cell populations to different gut microenvironments, suggesting that the microbiome may play a role (Bergsbaken and Bevan, 2015; Bergsbaken et al., 2017). Studies from our lab and others have identified that changes in the microbiome can influence the course of leishmania infection (Naik et al., 2015, 2012; Gimblet et al., 2017), and further identified that skin inflammation can tune commensal specific resident T cell responses (Naik et al., 2012). Whether or not the skin microbiota influences leishmania-specific T_{RM} cell responses remains to be defined, though preliminary data from our lab indicate that germ-free mice have similar frequencies of T_{RM} cells in the skin at the peak of infection (data not shown).

Finally, the distribution, composition, and size of T_{RM} cell niches in each tissue remain to be understood. The studies that have been done suggest a high degree of variability based on infection, tissue, and cell type. Studies in the skin initially suggested that CD8+ cells are much more likely to be confined to epidermal niches, while CD4+ cells are distributed within the dermis (Gebhardt et al., 2011). Follow up studies have identified a significant role for hair follicles and hair follicle-derived signals (Adachi et al., 2015; Collins et al., 2016), though these data remain to be confirmed in humans. Further, there appear to be differential signals required for the retention of T_{RM} cells in skin and the FRT (Schenkel et al., 2016). HSV infection in the female reproductive tract appears to generate memory lymphocyte clusters, in which myeloid cells and T_{RM} cells cohabitate and interact to sustain one another (Iijima and Iwasaki, 2014). T_{RM} cells in the skin similarly demonstrate clustering behavior in which CD4+, CD8+, and CD11c+ cells cluster together, and surprisingly CCL5+ from CD8+ cells is required for CD4+ T_{RM} cell retention (Collins et al., 2016). One of the least studied aspects of T_{RM} cells is the potential size of the T_{RM} niche. One study in the skin suggested that CD8+ T_{RM} cells compete with epidermal DETC cells for niche space (Zaid et al., 2014). Our data from figure 4.3 and data from the female reproductive tract and salivary glands (Schenkel et al., 2016) suggest that tissue resident immune responses can be boosted by repeated antigen exposure, suggesting that the niche can be expanded, though the upper limit remains to be defined.

5.6 Difficulties associated with studying T_{RM} cells

As the field of T_{RM} cells continues to move forward, it will be important to overcome a number of difficulties associated with studying T_{RM} cells. While multi-parametric flow

cytometry is a very useful technique for studying specific subsets of cells, the required digestion of tissue involved with sample preparation can result in a massive loss of cell number (Steinert et al., 2015), as well as a requisite loss of information regarding the position of cells within tissue. Increasingly, fluorescent imaging is being used to combat these issues, although there are technical difficulties associated with this technique as well, as it is often more difficult to stain for a large number of markers and to determine co-expression patterns. The continued development of cell-extraction and imaging techniques could greatly enhance the capacity to study T_{RM} cells.

The lack of a definitive signature for T_{RM} cells also makes it difficult to study these rare populations. Studies are often done by transferring transgenic T cells specific for one epitope of a pathogen, challenging, and analyzing those transferred cells remaining in the tissue after a period of time. This approach has the caveat that the response is monoclonal, and may not be representative of what is happening during natural infection. We were able to examine polyclonal responses in our studies, but this required the *ex vivo* restimulation of cells, which has the potential to skew any phenotypic markers. A more definitive signature for T_{RM} cells would help combat this issue, as it would allow T_{RM} cells to be identified by markers alone and not require additional manipulation.

A related issue critical for the study of T_{RM} cells is differentiating them from their circulating counterparts. Our group and others have taken great experimental lengths to distinguish these populations, which often requires techniques such as transplantation of tissue or parabiotic surgery. Again, further delineation of what defines a T_{RM} cell may help alleviate some of these issues. Intravascular labeling of cells in direct blood contact

(Anderson et al., 2014) has also proven a useful resource for distinguishing blood and tissue associated cells, though it may not be as effective for highly vascularized tissues, and cannot distinguish true T_{RM} cells from cells that are temporarily in tissue. *In vivo* imaging with 2-photon microscopy may also allow for nonbiased analysis of migratory and non-migratory T cell populations, though the length of time for which one can run these analyses is limited. Fortunately, for some questions it is probably not critical to distinguish whether a population of T cells in tissue is truly resident, but rather whether or not they contribute to the resolution or pathogenesis of disease.

To that end, the data presented here identify skin-resident CD4+ T cells as critical mediators of protection against cutaneous leishmaniasis. In addition to clarifying multiple mechanisms by which these cells protect, we have identified factors associated with the formation of this population, and even demonstrated at least one way in which they might be generated in a vaccine. Despite the many difficulties associated with studying tissue-resident populations, it will be important to continue to study these cells not only as a means of understanding protection against leishmaniasis, but also as a general tool for understanding how CD4+ T cell responses are formed and maintained in tissue, and the role that these cells play in establishing protective immunity.

5.7 Materials and Methods

DNA Electroporation

25 µg of DNA plasmid encoding HIV-env was injected intramuscularly into the tibialis anterior or intradermally into the side skin of C57BL/6 mice. 14 days later, the vaccination was repeated. 10 days after the second vaccination, mice were sacrificed

and spleen, skin from the vaccination site, and skin from the opposite side were harvested. Spleen and skin were restimulated with overlapping 11mer peptide pools, and IFNγ producing cells were measured by Elispot or flow cytometry.

Cell Sort and qPCR

CD4+ cells from the DLN or primary ear of C57BL/6 mice 7 weeks post *L. major* infection were sorted based on CD44 expression (DLN) or CD69 expression (Ear) by FACS Aria into RLT buffer (Qiagen). RNA was isolated using RNeasy plus pico kit (Qiagen), and qPCR was performed for Hobit and Blimp-1 using primers (F: 5'-CTCAGCCACTTGCAGACTCA-3', R: 5'-CTGTCGGTGGAGGCTTTGTA-3'), and (F: 5'-TTCTCTTGGAAAAACGTGTGGG-3', R: 5'-GGAGCCGGAGCTAGACTTG-3') respectively.

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